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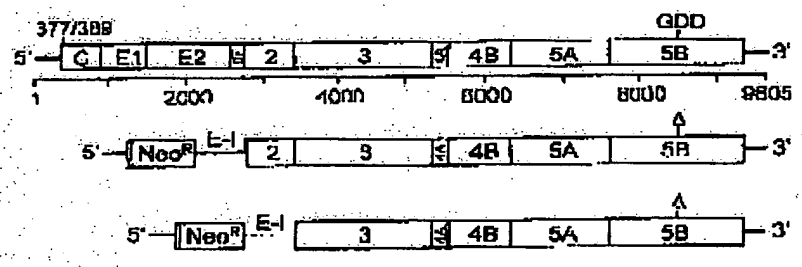
(12) **Patent Application:** (11) **CA 2303526**

(54) HEPATITIS C VIRUS CELL CULTURE SYSTEM

(54) SYSTEME DE CULTURE DU VIRUS DE L'HEPATITE C

Representative Drawing:



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ABSTRACT:

The hepatitis C virus (HCV) cell culture system according to the invention consists of human hepatoma cells, which are transfected with a HCV-RNA construct, that comprises the HCV specific RNA segments 5' NTR, NS3, NS4A, NS4B,
NS5A, NS5B, and 3' NTR as well as a minimum of one marker gene for selection (selection gene).

CLAIMS: Show all claims

*** Note: Data on abstracts and claims is shown in the official language in which it was submitted.

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C12N 5/10
C12N 15/51

Patent Cooperation Treaty (PCT): **No**

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Availability of licence: **N/A**

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CC DocNum	KD PubDate	CC AppNum	KD AppDate
☐ AT 236988	E 20030415	AT 105929	EP 20000323
		DE 19915178	A 19990403
☐ AU 200025180	A5 20001019	AU 25180	A 20000331
		DE 19915178	A 19990403
☐ CA 2303526	AA 20001003	CA 2303526	A 20000331
		DE 19915178	A 19990403
☐ DE 19915178	A1 20001005	DE 19915178	A 19990403
		DE 19915178	A 19990403
☐ DE 50001673	C0 20030515	DE 19915178	A 19990403
		DE 50001673	A 20000323
		DE 50001673	A 20000323
☐ DK 1043399	T3 20030728	DE 19915178	A 19990403
		DK 105929	A 20000323
☐ EP 1043399	A2 20001011	DE 19915178	A 19990403
		EP 105929	A 20000323
☐ EP 1043399	B1 20030409	DE 19915178	A 19990403
		EP 105929	A 20000323
☐ EP 1043399	A3 20020508	DE 19915178	A 19990403
		EP 105929	A 20000323
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10 Publications found.			

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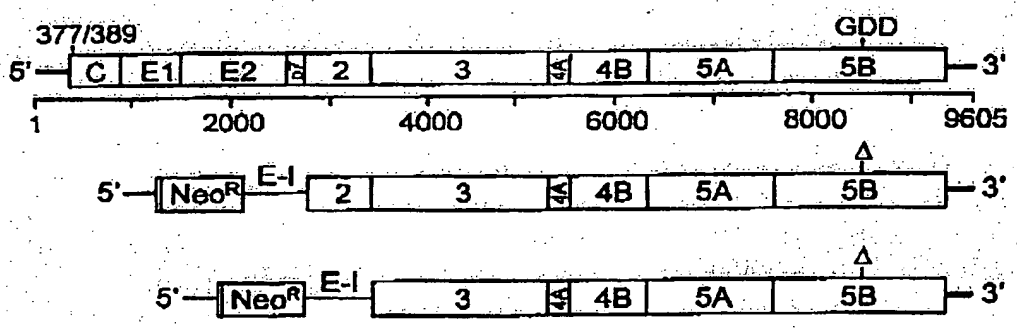
(12) (19) (CA) **Demande-Application**

(21) (A1) **2,303,526**
(22) 2000/03/31
(43) 2000/10/03

(72) BARTENSCHLAGER, RALF, DE
(71) BARTENSCHLAGER, RALF, DE

(51) Int.Cl.[7] C12N 7/02, C12N 15/51, C12N 5/10
(30) 1999/04/03 (199 15 178.4) DE

(54) **SYSTEME DE CULTURE DU VIRUS DE L'HEPATITE C**
(54) **HEPATITIS C VIRUS CELL CULTURE SYSTEM**



(57) The hepatitis C virus (HCV) cell culture system according to the invention consists of human hepatoma cells, which are transfected with a HCV-RNA construct, that comprises the HCV specific RNA segments 5' NTR, NS3, NS4A, NS4B, NS5A, NS5B, and 3' NTR as well as a minimum of one marker gene for selection (selection gene).

Hepatitis C Virus Cell Culture System

ABSTRACT

The hepatitis C virus (HCV) cell culture system according to the invention consists of human hepatoma cells, which are transfected with a HCV-RNA construct, that comprises the HCV specific RNA segments 5' NTR, NS3, NS4A, NS4B, NS5A, NS5B, and 3' NTR as well as a minimum of one marker gene for selection (selection gene).

CLAIMS

1. A hepatitis C virus (HCV) cell culture system, which comprises mainly eukaryotic cells containing transfected HCV specific genetic material, characterized in that,
the eukaryotic cells are human hepatoma cells and the transfected HCV specific genetic material is a HCV-RNA construct, which comprises the HCV specific RNA segments 5' NTR, NS3, NS4A, NS4B, NS5A, NS5B, and 3' NTR as well as an additional marker gene for selection (selection gene).

2. A cell culture system according to Claim 1, characterized in that,
the hepatoma cells are derived from a usual hepatoma cell line.

3. A cell culture system according to Claim 1, characterized in that,
the hepatoma cells are extracted from a primary hepatoma cell culture.

4. A HCV-RNA construct, characterized in that,
it comprises the HCV specific RNA segments 5' NTR, NS3, NS4A, NS4B, NS5A, NS5B, and 3' NTR as well as an additional marker gene for selection (selection gene).

5. A HCV-RNA construct according to Claim 4, characterized in that,
it comprises one of the nucleotide sequences illustrated in the sequence protocols SEQ ID NO: 1 to SEQ ID NO: 11.

6. A HCV-RNA construct according to Claim 4, characterized in that,

the 3' NTR has a nucleotide sequence, which has been selected from the group of nucleotide sequences (a) to (i) listed in the following:

(a) ACGGGGAGCTAAACACTCCAGGCCAATAGGCCATCCTGTTTTTTTTTTTA
GCTTTTTTTTTTTTCTTTTTTTTTGAGAGAGAGAGTCTCACTCTGTTGCC
CAGACTGGAGT

(b) ACGGGGAGCTAAACACTCCAGGCCAATAGGCCATCCTGTTTTTTTTTTTA
GTCTTTTTTTTTTCTTTTTTTTGAGAGAGAGAGTCTCACTCTGTTGCCCA
GACTGGAGC

(c) ACGGGGAGCTAAACACTCCAGGCCAATAGGCCATCCTGTTTTTTTTTAAT
CTTTTTTTTTTTCTTTTTTTTTGAGAGAGAGAGTCTCACTCTGTTGCCCA
GACTGCAGC

(d) ACGGGGAGCTAAACACTCCAGGCCAATAGGCCATCCTGTTTTTTTTTTTT
AGTCTTTTTTTTTTTCTTTTTTTTTGAGAGAGAGAGTCTCACTCTGTTGC
CCAGACTGGAGT

(e) ACGGGGAGCTAAACACTCCAGGCCAATAGGCCATCCTGTTTTTTTTTTTA
GTCTTTTTTTTTTTTCTTTTTTTTTGAGAGAGAGAGTCTCACTCTGTTGC
CCAGACTGGAGT

(f) ACGGGGAGCTAAACACTCCAGGCCAATAGGCCATCCTGTTTTTTTTTTTA
GTCTTTTTTTTTTTTCTTTTTTTTTGAGAGAGAGAGTCTCACTCTGTTG
CCCAGACTGGAGT

(g) ACGGGGAGCTAAACACTCCAGGCCAATAGGCCATCCTGTTTTTTTTTTTA
GTCTTTTTTTTTTTCTTTTTTTTTGAGAGAGAGAGTCTCACTCTGTTGCC
CAGACTGGAGT

(h) ACGGGGAGCTAAACACTCCAGGCCAATAGGCCATCCTGTTTTTTTTTTTT
TAATCTTTTTTTTTTTTTCCTTTTTTTGAGAGAGAGAGTCTCACTCTGT
TGCCCAGACTGGAGT

(i) ACGGGGAGCTAAACACTCCAGGCCAATAGGCCATCCTGTTTTTTTTTTTT
AATCTTTTTTTTTTTTTTCTTTTTTTTTTGAGAGAGAGAGTCTCACTCTG
TTGCCCAGACTGGAGT

7. A HCV-RNA construct according to one of the Claims 4 to 6, characterized in that,
the marker gene for selection is a resistance gene and preferably an antibiotic resistance gene.

8. A HCV-RNA construct according to one of the Claims 4 to 6, characterized in that,
the marker gene for selection is a neomycin phosphotransferase gene.

9. A HCV-RNA construct according to one of the Claims 4 to 8, characterized in that,
the marker gene for selection is integrated in the HCV-RNA downstream from the 5' NTR.

10. A HCV-RNA construct according to one of the Claims 4 to 83, characterized in that,
the marker gene for selection is linked to the HCV-RNA via a ribozyme or a recognition sequence for a ribozyme.

11. A HCV-RNA construct according to one of the Claims 4 to 10, characterized in that,
it has an integrated reporter gene.

12. A HCV-RNA construct according to Claim 11, characterized in that,
the reporter gene is a gene from the group of the luciferase genes, the CAT gene (chloramphenicol acetyl transferase gene), the lacZ gene (beta galactosidase gene), the GFP gene (green fluorescence protein gene), the GUS gene (glucuronidase gene) and the SEAP gene (secreted alkaline phosphatase gene).

13. A HCV-RNA construct according to one of the Claims 4 to 11, characterized in that,
their replication has an influence on the expression of a (cellular) surrogate marker gene.

14. A HCV-RNA construct according to one of the Claims 11 to 13, characterized in that,
the resistance gene is cloned into the open reading frame of the HCV-RNA in such a way that it will only be transferred to an active form after proteolytic processing.

15. A HCV-RNA construct according to one of the Claims 11 to 14, characterized in that,
the reporter gene and the marker gene for selection are arranged in the construct in such a way, that they are expressed as a fusion protein.

16. A cell culture system according to one of the Claims 1 to 3, characterized in that,
the HCV-RNA construct is a construct according to at least one of the Claims 4 to 15.

17. A cell culture system according to Claim 1, characterized in that,
the cells containing the HCV-RNA construct are lodged at the DSMZ, Braunschweig, Germany under the deposit number DSM ACC2394 (laboratory name HuBl 9-13).

18. A use of a cell culture system according to one of the Claims 1 to 3 or 16 to 17 and/or a HCV-RNA construct according to one of the Claims 4 to 15 for the production and/or evaluation and/or testing of treatments and/or diagnostics for the treatment of HCV infections in particular.

19. A use of a cell culture system according to one of the Claims 1 to 3 or 16 to 17 and/or a HCV-RNA construct according to one of the Claims 4 to 15 for the production of a vaccine against HCV infections.

20. A use a HCV-RNA construct according to one of the Claims 4 to 15 for the production of a liver cell specific vector in gene therapy.

21. A HCV-RNA construct according to one of the Claims 4 to 15, characterized in that,
it has an integrated foreign gene and can be used to transfect this foreign gene in a target cell, which can be used to express this foreign gene.

22. A process for the production of cell culture adapted mutants of a HCV-RNA construct according to one of the claims 4 to 15, in which the mutants have increased replication efficiency compared to the HCV-RNA construct,
characterized in that,
a cell culture system according to claim 1, in which the transfected HCV specific genetic material is a HCV-RNA construct with a selection gene according to one of the claims 4 to 15, is cultivated on/in the selection medium corresponding to the selection gene, that the cell clones created are collected, and that the HCV-RNA constructs or parts thereof are isolated from these cell clones.

23. A process according to claim 22, characterized in that,
the isolated HCV-RNA constructs are passaged at least one more time. That is, they are transfected in cells of a cell culture system according to claim 1. The cell culture system of claim 1 thereby obtained, in which the transfected HCV specific genetic material is

the isolated HCV-RNA construct with a selection gene, is cultivated on/in the selection medium corresponding to the selection gene, the cell clones created are collected, and the HCV-RNA constructs are isolated from these cell clones.

24. A process for the production of mutants of a HCV full-length genome or of a HCV subgenome or any HCV construct with increased replication efficiency in comparison to the original HCV full-length genome or subgenome or HCV-RNA construct,
characterized in that,
a cell culture adapted mutant of a HCV-RNA construct is produced and isolated by a process according to claim 22 or 23,
the nucleotide and amino acid sequence of these mutants is determined and the type, number and positions of the nucleotide and amino acid mutations is determined by comparison with the nucleotide and amino acid sequence of the original HCV-RNA construct, and these mutations are introduced in an (isolated) HCV full-length genome or a HCV subgenome or any HCV-RNA construct, either by site-directed mutagenesis or by exchange of fragments containing the relevant mutations

25. A cell culture adapted HCV-RNA construct with high replication efficiency,
characterized in that,
it is derivable from a HCV-RNA construct according to one of the claims 4 to 15 through nucleotide and/or amino acid mutations and that it is obtainable with a process according to one of the claims 22 to 24.

26. A cell culture adapted HCV-RNA construct according to claim 25,
characterized in that,

it carries one or several of the following amino acid exchanges: 1283 arg -> gly and/or 1383 glu -> ala and/or 1577 lys -> arg and/or 1609 lys -> glu and/or 1936 pro -> ser and/or 2163 glu -> gly and/or 2330 lys -> glu and/or 2442 ile -> val.

27. A cell culture adapted HCV-RNA construct according to claim 25 or 26,
characterized in that,
it displays one or several of the nucleotide and/or amino acid exchanges shown in Table 3, where Table 3 is an integral part of this claim.

28. Cell culture adapted mutant of a HCV-RNA construct or of a HCV full-length genome with increased replication efficiency in comparison to the original HCV-RNA construct or original HCV full-length genome, characterized in that,
it is obtainable with a process in which the number and type of mutations in a cell culture adapted HCV-RNA construct according to claim 24 are determined through sequence analysis and sequence comparison, and these mutations are introduced in a HCV-RNA construct, particularly in a HCV-RNA construct according to one of the claims 4 to 15, or in an (isolated) HCV-RNA full-length genome, either by targeted mutagenesis or by exchange of sequence sections containing the relevant mutations.

29. Hepatitis C virus particles or virus-like particles, characterized in that,
they are obtainable by a process according to one of the claims 22 - 24.

30. Cells infected with Hepatitis C virus particles or virus-like particles, according to claim 29.

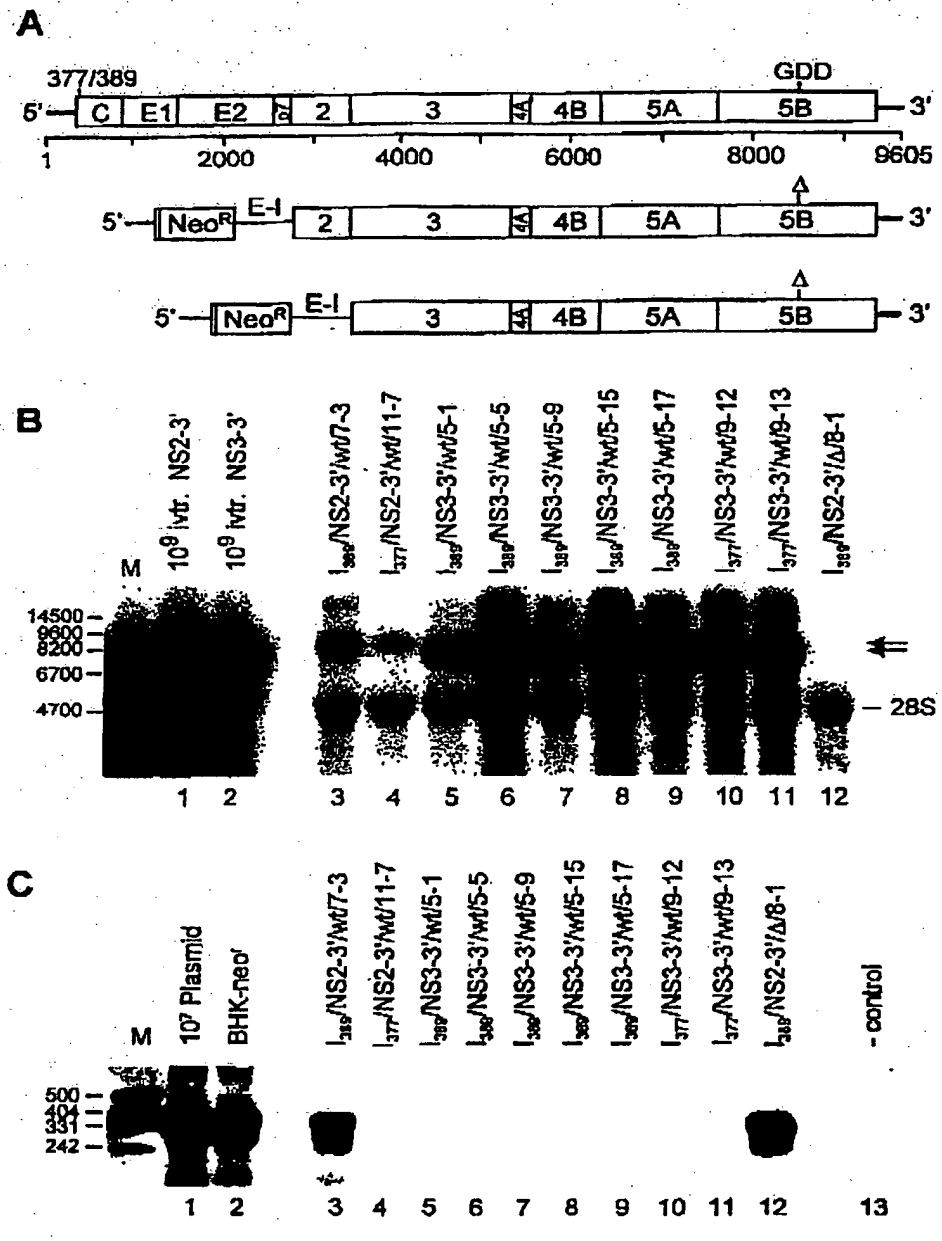

A
377/389
GDD
5'
C
E1
E2
p7
2
3
4A
4B
5A
5B
3'
1
2000
4000
6000
8000
9605
Neo^R
E-I
B
M
10^9 ivtr. NS2-3'
10^9 ivtr. NS3-3'
I389/NS2-3'/wt/7-3
I377/NS2-3'/wt/11-7
I389/NS3-3'/wt/5-1
I389/NS3-3'/wt/5-5
I389/NS3-3'/wt/5-9
I389/NS3-3'/wt/5-15
I389/NS3-3'/wt/5-17
I389/NS3-3'/wt/9-12
I377/NS3-3'/wt/9-13
I389/NS2-3'/Δ/8-1
14500
9600
8200
6700
4700
28S
1 2 3 4 5 6 7 8 9 10 11 12
C
M
10^7 Plasmid
BHK-neo'
I389/NS2-3'/wt/7-3
I377/NS2-3'/wt/11-7
I389/NS3-3'/wt/5-1
I389/NS3-3'/wt/5-5
I389/NS3-3'/wt/5-9
I389/NS3-3'/wt/5-15
I389/NS3-3'/wt/5-17
I377/NS3-3'/wt/9-12
I377/NS3-3'/wt/9-13
I389/NS2-3'/Δ/8-1
- control
500
404
344
242
1 2 3 4 5 6 7 8 9 10 11 12 13

Fig. 1

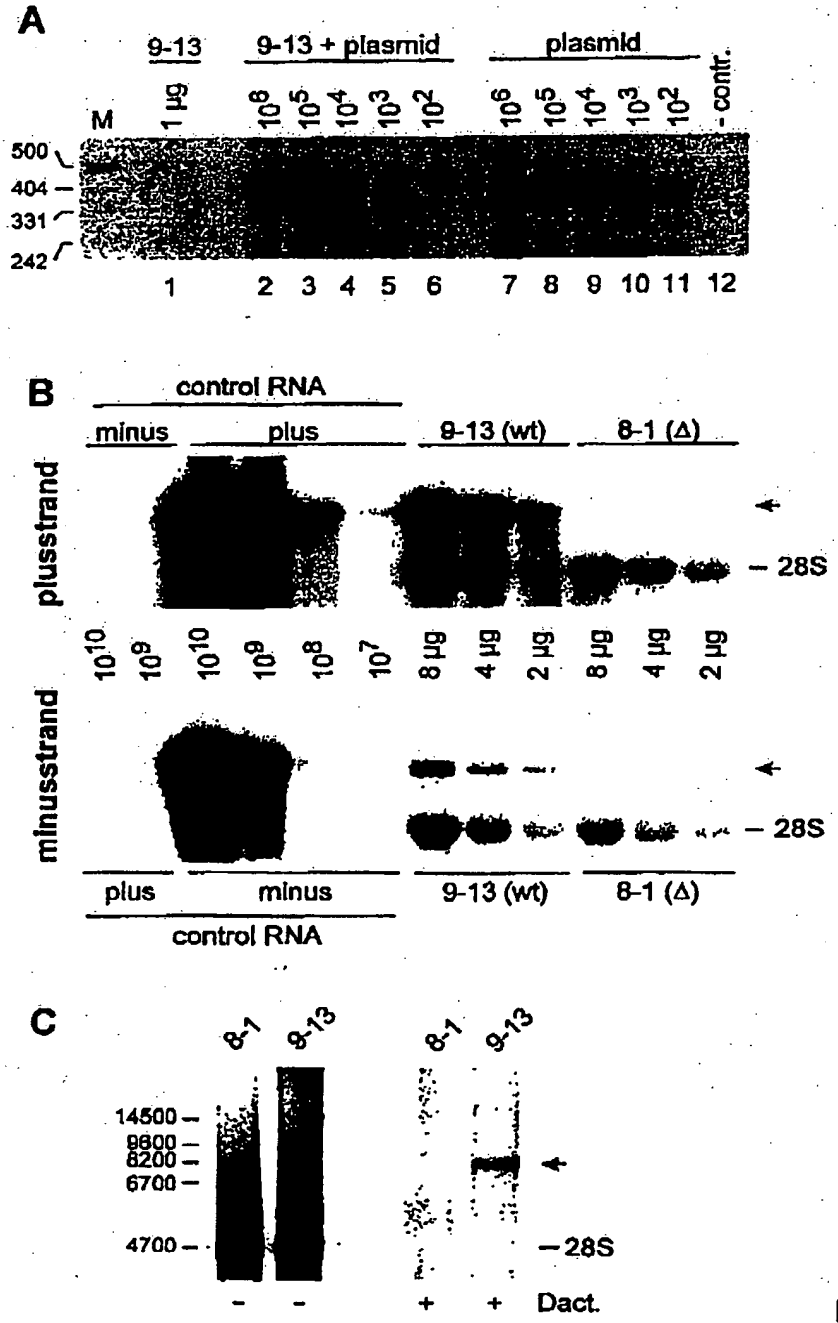

A
9-13
9-13 + plasmid
plasmid
M
1 µg
10^8 10^5 10^4 10^3 10^2
10^6 10^5 10^4 10^3 10^2
- contr.
500
404
331
242
1 2 3 4 5 6 7 8 9 10 11 12
B
control RNA
minus
plus
9-13 (wt)
8-1 (Δ)
plusstrand
– 28S
10^10 10^9 10^10 10^9 10^8 10^7 8 µg 4 µg 2 µg 8 µg 4 µg 2 µg
minusstrand
– 28S
plus
minus
9-13 (wt)
8-1 (Δ)
control RNA
C
8-1
9-13
8-1
9-13
14500
9800
8200
6700
4700
–28S
- - + + Dact.

Fig. 2

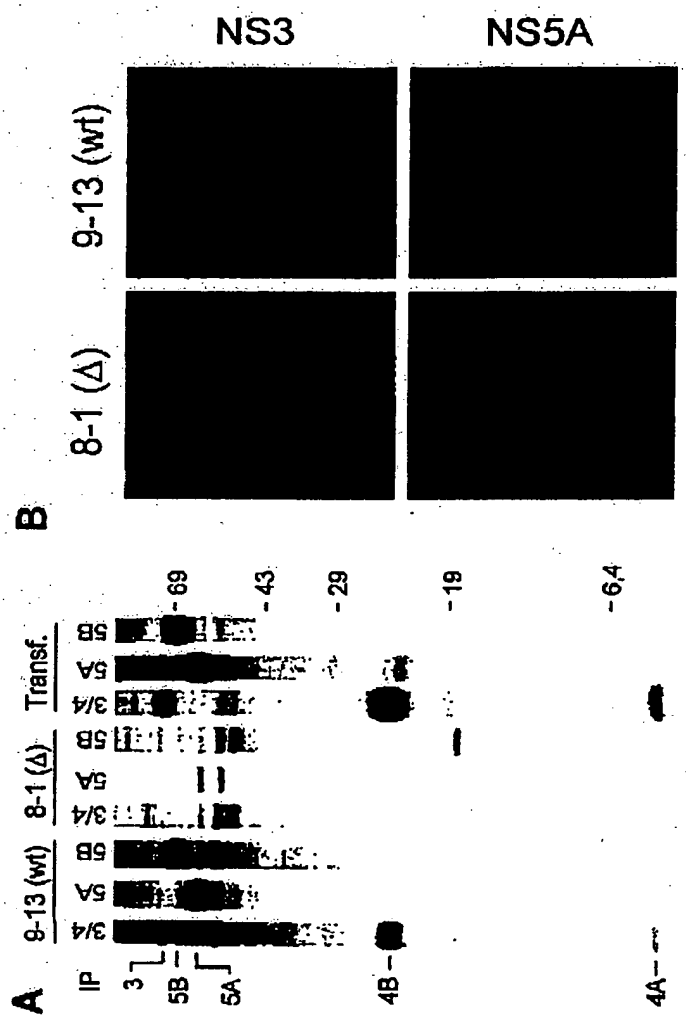

NS3
NS5A
9-13 (wt)
8-1 (Δ)
B
A
IP
9-13 (wt)
8-1 (Δ)
Transf.
3/4
5A
5B
3
5B
5A
4B
4A
-69
-43
-29
-19
-6,4
1 2 3 4 5 6 7 8 9

Fig. 3

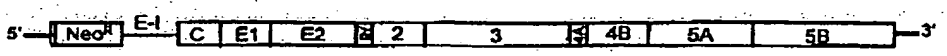

5'
Neo^R
E-I
C
E1
E2
2
3
4B
5A
5B
3'

Fig. 4

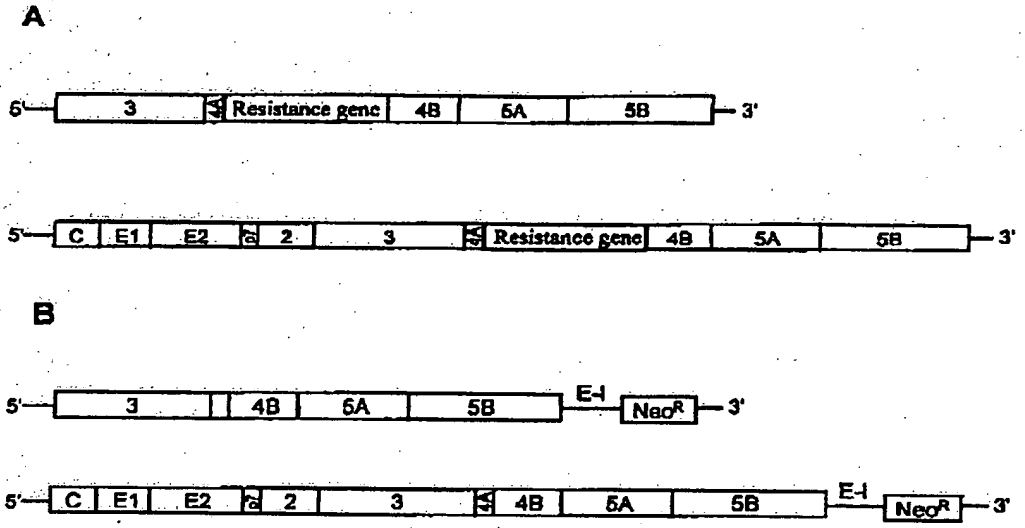

A
5'
3
Resistance gene
4B
5A
5B
3'
5'
C
E1
E2
2
3
Resistance gene
4B
5A
5B
3'
B
5'
3
4B
5A
5B
E-I
Neo^R
3'
5'
C
E1
E2
2
3
4B
5A
5B
E-I
Neo^R
3'

Fig. 5

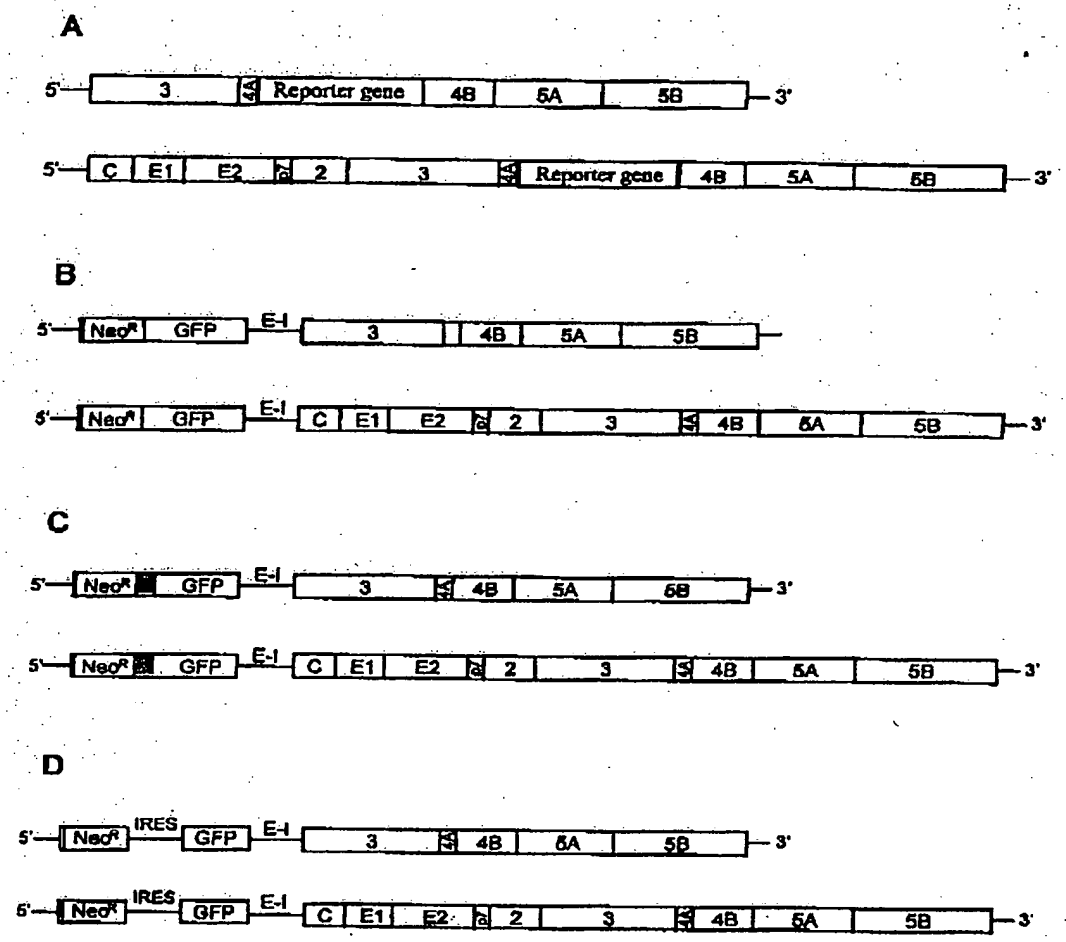

A
5'
3
Reporter gene
4B
5A
5B
3'
5'
C
E1
E2
2
3
Reporter gene
4B
5A
5B
3'
B
5'
Neo^R
GFP
E-I
3
4B
5A
5B
5'
Neo^R
GFP
E-I
C
E1
E2
2
3
4B
5A
5B
3'
C
5'
Neo^R
GFP
E-I
3
4B
5A
5B
3'
5'
Neo^R
GFP
E-I
C
E1
E2
2
3
4B
5A
5B
3'
D
5'
Neo^R
IRES
GFP
E-I
3
4B
5A
5B
3'
5'
Neo^R
IRES
GFP
E-I
C
E1
E2
2
3
4B
5A
5B
3'

Fig. 6

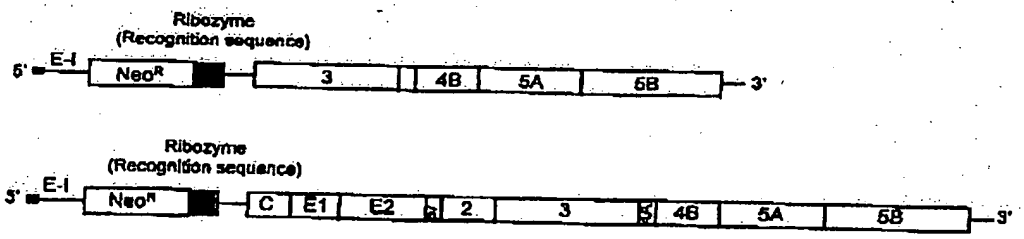

Ribozyme
(Recognition sequence)
5'
E-I
Neo^R
3
4B
5A
5B
3'
Ribozyme
(Recognition sequence)
5'
E-I
Neo^R
C
E1
E2
p7
2
3
NS4A
4B
5A
5B
3'

Fig. 7

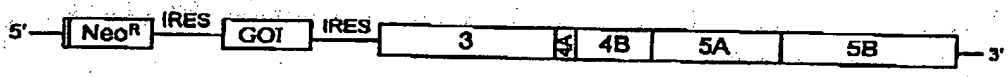

5'
Neo^R
IRES
GOI
IRES
3
NS4A
4B
5A
5B
3'

Fig. 8

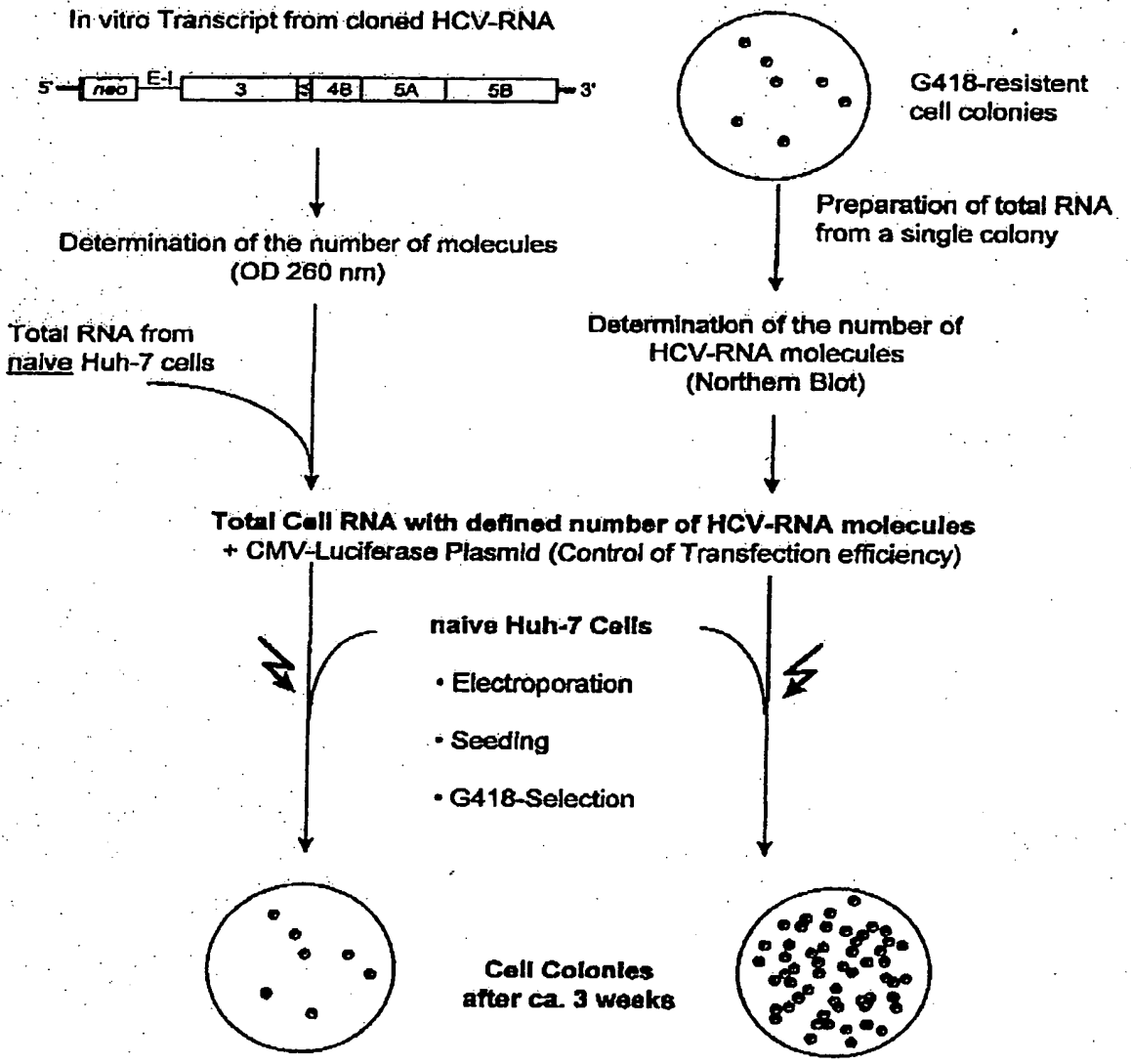

In vitro Transcript from cloned HCV-RNA
5'
neo
E-I
3
4B
5A
5B
3'
G418-resistent cell colonies
Determination of the number of molecules (OD 260 nm)
Preparation of total RNA from a single colony
Total RNA from naive Huh-7 cells
Determination of the number of HCV-RNA molecules (Northern Blot)
Total Cell RNA with defined number of HCV-RNA molecules + CMV-Luciferase Plasmid (Control of Transfection efficiency)
naive Huh-7 Cells
• Electroporation
• Seeding
• G418-Selection
Cell Colonies after ca. 3 weeks

Fig. 9

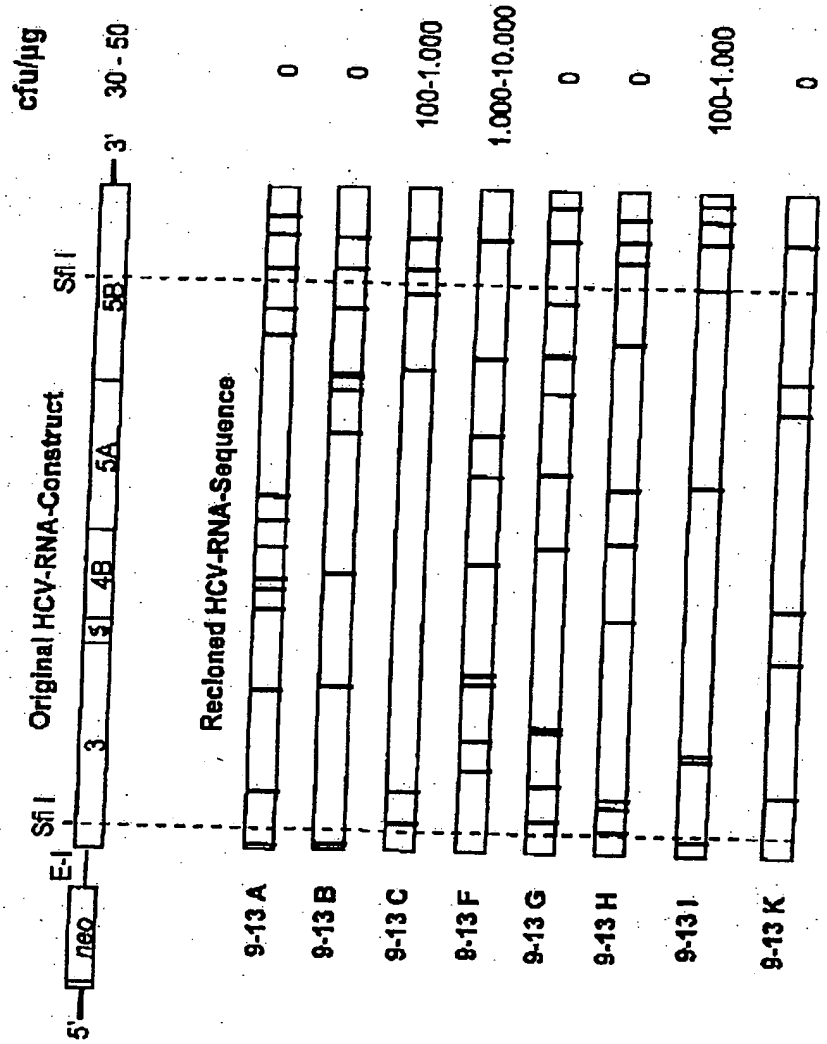
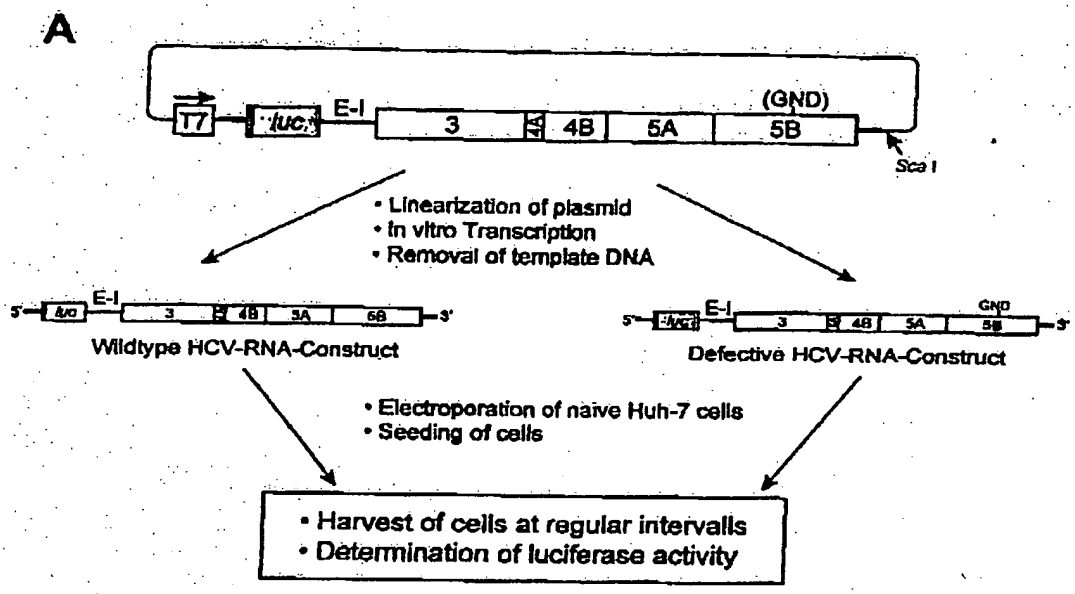

cfu/µg
30 - 50
3'
Sfi I
5B
5A
4B
3
Sfi I
E-I
neo
5'
Original HCV-RNA-Construct
Recloned HCV-RNA-Sequence
0
0
100-1.000
1.000-10.000
0
0
100-1.000
0
9-13 A
9-13 B
9-13 C
8-13 F
9-13 G
9-13 H
9-13 I
9-13 K

Fig. 10


A
T7
luc
E-I
3
4B
5A
5B
(GND)
Sca I
• Linearization of plasmid
• In vitro Transcription
• Removal of template DNA
Wildtype HCV-RNA-Construct
Defective HCV-RNA-Construct
• Electroporation of naive Huh-7 cells
• Seeding of cells
• Harvest of cells at regular intervalls
• Determination of luciferase activity

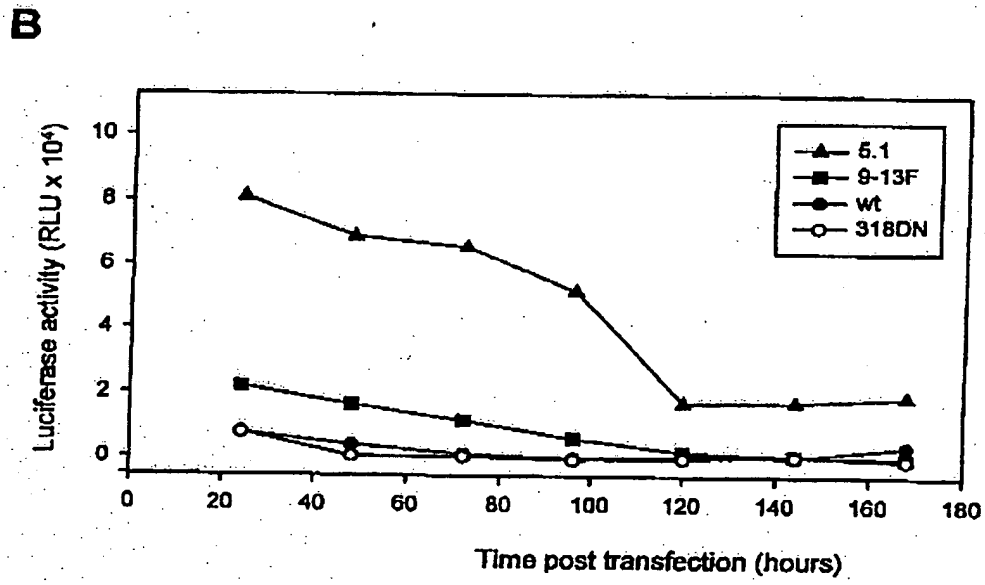
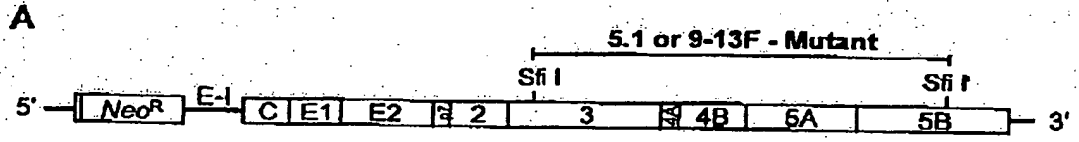

B
Luciferase activity (RLU x 10⁴)
Time post transfection (hours)
5.1
9-13F
wt
318DN
0
2
4
6
8
10
0
20
40
60
80
100
120
140
160
180

Fig. 11

A



B

G418-Concentration:	500 µg/ml	250 µg/ml	100 µg/ml
Number of Cell Colonies:	1	23	75

C

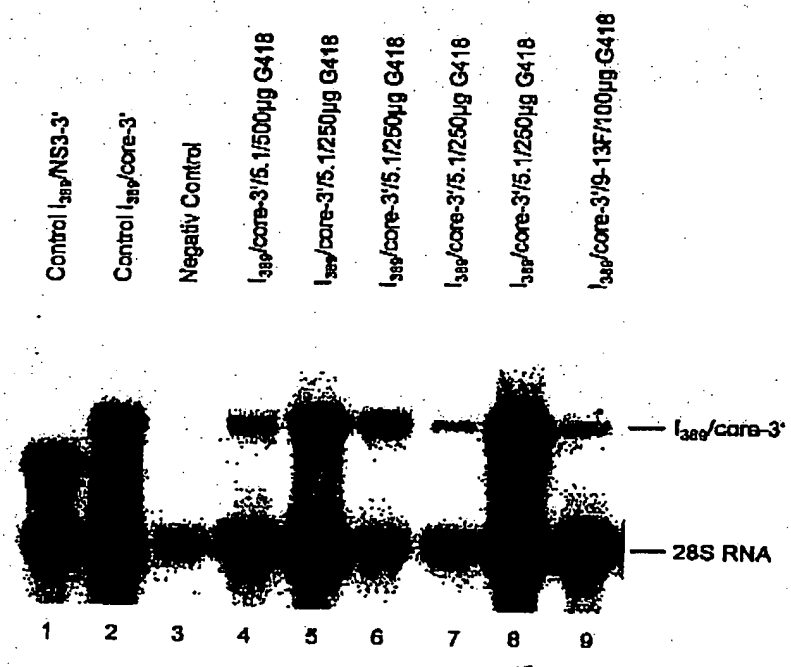


Fig. 12

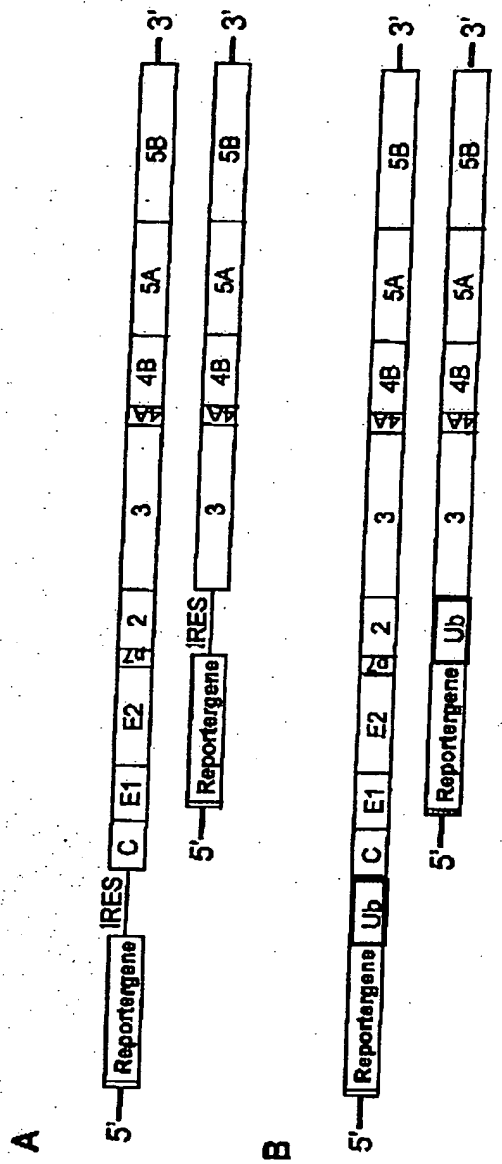


Fig. 13

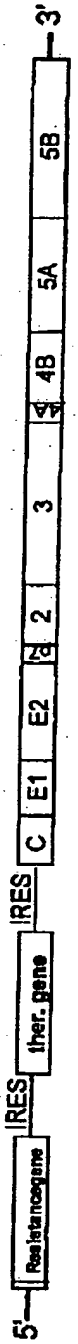


Fig. 14

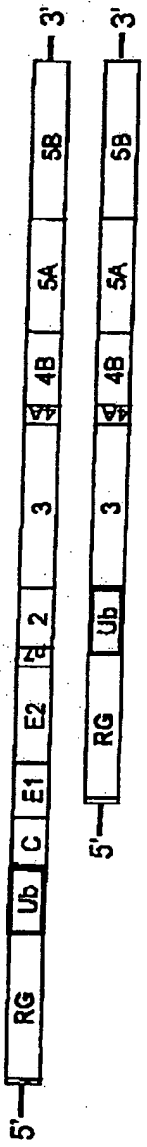


Fig. 15

90°C
180°C
1 / 98

CA 02303526 2000-03-31

Hepatitis C Virus Cell Culture System

DESCRIPTION

The invention relates to a hepatitis C virus (HCV) cell culture system, which comprises mainly eukaryotic cells containing transfected HCV specific genetic material, which means they are transfected with HCV specific genetic material.

The hepatitis C virus (HCV) is one of the main causes worldwide of chronic and sporadic liver diseases. The history of most HCV infections does not involve any obvious clinical signs, but 80 - 90 % of the infected people become chronic carriers of the virus and 50 % of these chronic carriers of the virus develop chronic hepatitis with different degrees of severity. Approx. 20 % of the chronically infected develop a cirrhosis of the liver over 10 to 20 years, based on what a primary hepatocellular carcinoma can develop. Nowadays chronic hepatitis C is the main indication for liver transplantation. A specific therapy does not exist until now. The only therapy currently available is high-dose administration of Interferon alpha or a combination of Interferon alpha and the purine nucleoside analogue Ribavirin. However, only approx. 60 % of all treated persons respond to this therapy and with these, a new viraemia occurs in more than half of all cases after the discontinuation of the treatment.

Due to the high prevalence, especially in industrialized countries, the serious effects of chronic infections and the non-existence of a specific therapy, the development of a HCV specific chemotherapy is an important goal of pharmaceutical research and development. The main problem lies in the previous lack of a suitable cell culture system, which enables the study of virus replication and pathogenesis in eukaryotic cells.

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Due to the high prevalence, especially in industrialized countries, the serious effects of chronic infections and the non-existence of a specific therapy, the development of a HCV specific chemotherapy is an important goal of pharmaceutical research and development. The main problem lies in the previous lack of a suitable cell culture system, which enables the study of virus replication and pathogenesis in eukaryotic cells.

Due to the small amount of virus in blood or tissue, the lack of suitable cell culture systems or animal models (the chimpanzee is still the only possible experimental animal) as well as the lack of efficient systems for producing virus-like particles, it was not possible up to now, to analyze the molecular composition of the HCV particle in-depth and to solve it. The information currently available can be summarized as follows: HCV is an enveloped plus-strand RNA virus with a particle diameter of 50 - 60 nm and a medium density of 1.03 - 1.1 g/ml. It was molecularly cloned and characterized for the first time in 1989 (Choo et al., 1989, Science, 244, 359 - 362). The HCV-RNA has a length of approx. 9.6 kb (= 9600 nucleotides), a positive polarity and comprises one open reading frame (ORF), which encodes a linear polyprotein of approx. 3010 amino acids (see Rice 1996, in Virology, B. N. Fields, D. M. Knipe, P. M. Howley, Eds. (Lippincott-Raven, Philadelphia, PA, 1996), vol. 1, pp. 931 - 960; Clarke 1997, *J. Gen. Virol.* 78, 2397; and Bartenschlager 1997, *Intervirology* 40, 378 and see Fig. 1 A). During the replication of the virus the polyprotein is cleaved into the mature and functionally active proteins by cellular and viral proteases.

Within the polyprotein the proteins are arranged as follows (from the amino- to the carboxy terminus): Core-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B. The core protein is the main component of the nucleocapsid. The glycoproteins E1 and E2 are transmembrane proteins and the main components of the viral envelope. They probably play an important role during the attachement of the virus to the host cell. These three proteins core, E1, and E2 constitute the viral particle and are therefore called structural proteins. The function of the protein p7 is still not clear. The protein NS2 is probably the catalytic domain of the NS2-3 protease, which is responsible for the processing between the proteins NS2 and NS3. The protein NS3 has two functions, one is a

protease activity in the amino terminal domain, which is essential for the polyprotein processing, and the other a NTPase/helicase function in the carboxy terminal domain, which is probably important during the replication of the viral RNA. The protein NS4A is a co-factor of the NS3 protease. The function of the protein NS4B is unknown.

The open reading frame is flanked on its 5' end by a non-translated region (NTR) approx. 340 nucleotides in length, which functions as the internal ribosome entry site (IRES), and on its 3' end by a NTR approx. 230 nucleotides in length, which is most likely important for the genome replication. A 3' NTR such as this is the object of patent application PCT/US 96/14033. The structural proteins in the amino terminal quarter of the polyprotein are cleaved by host cell signal peptidase. The non-structural proteins (NS) 2 to (NS) 5B are processed by two viral enzymes, namely the NS2-3 and the NS3/4A protease. The NS3/4A protease is required for all cleavages beyond the carboxy terminus of NS3. The function of NS4B is unknown. NS5A, a highly phosphorylated protein, seems to be responsible for the Interferon resistance of various HCV genotypes (see Enomoto et al. 1995, *J. Clin. Invest.* 96, 224; Enomoto et al. 1996, *N. Engl. J. Med.* 334, 77; Gale Jr. et al. 1997, *Virology* 230, 217; Kaneko et al. 1994, *Biochem. Biophys. Res. Commun.* 205, 320; Reed et al., 1997, *J. Virol.* 71, 7187), and NS5B has been identified as the RNA-dependent RNA polymerase.

First diagnostic systems have been developed from these findings, which are either based on the detection of HCV specific antibodies in patient serum or the detection of HCV specific RNA using the reverse transcription polymerase chain reaction (RT-PCR), and which are (must be) routinely used with all blood and blood products and/or according to the regulations.

Since the first description of the genome in 1989 several partial and complete sequences of the HCV have been cloned and characterized using the PCR method. A comparison of these sequences shows a high variability of the viral genome in particular in the area of the NS5B gene, which eventually resulted in the classification of 6 genotypes, which are again subdivided into the subtypes a, b, and c.

The genomic variance is not evenly distributed over the genome. The 5'NTR and parts of the 3'NTR are highly conserved, while certain encoded sequences vary a lot, in particular the envelope proteins E1 and E2.

The cloned and characterized partial and complete sequences of the HCV genome have also been analyzed with regard to appropriate targets for a prospective antiviral therapy. In the course of this, three viral enzymes have been discovered, which may provide a possible target. These include (1) the NS3/4A protease complex, (2) the NS3 Helicase and (3) the NS5B RNA-dependent RNA polymerase. The NS3/4A protease complex and the NS3 Helicase have already been crystallized and their three-dimensional structure determined (Kim et al., 1996, Cell, 87,343; Yem et al., 1998, Protein Science, 7, 837; Love et al., 1996, Cell, 87, 311; Kim et al., 1998, Structure, 6, 89; Yao et al., 1997, Nature Structural Biology, 4, 463, Cho et al., 1998, J. Biol. Chem., 273, 15045). it has not been successful until now with the NS5B RNA-dependent RNA polymerase.

Even though important targets for the development of a therapy for chronic HCV infection have been defined with these enzymes and even though a worldwide intensive search for suitable inhibitors is ongoing with the aid of rational drug design as well as high throughput screening, the development of a therapy has one major deficiency, namely the lack of cell culture systems or simple animal models, which allow direct, reliable identification of HCV-RNA or

HCV antigens with simple methods which are common in the laboratory. The lack of these cell culture systems is also the main reason that to date the comprehension of HCV replication is still incomplete and mainly hypothetical.

Although according to the experts, a close evolutionary relationship exists between HCV and the flavi- and pestiviruses, and self-replicating RNAs have been described for these, which can be used for the replication in different cell lines with a relatively high yield, (see Khromykh et al., 1997, *J. Virol.* 71, 1497; Behrens et al., 1998, *J. Virol.* 72, 2364; Moser et al., 1998, *J. Virol.* 72, 5318), similar experiments with HCV have not been successful to date.

Although it is known from different publications that cell lines or primary cell cultures can be infected with high titre patient serum containing HCV, (Lanford et al. 1994, *Virology* 202, 606; Shimizu et al. 1993, *Proceedings of the National Academy of Sciences*, USA, 90, 6037 - 6041; Mizutani et al. 1996, *Journal of Virology*, 70, 7219 - 7223; M. Ikeda et al. 1998, *Virus Res.* 56, 157; Fournier et al. 1998, *J. Gen. Virol.* 79, 2376 and bibliographical references quoted in here; Ito et al. 1996, *Journal of General Virology*, 77, 1043 - 1054), these virus-infected cell lines or cell cultures do not allow the direct detection of HCV-RNA or HCV antigens. The viral RNA in these cells can not be detected in a Northern Blot (a standard method for the quantitative detection of RNA) or the viral protein in a Western Blot or with immunoprecipitation. It has only been possible to detect HCV replication with very costly and indirect methods. These disadvantageous facts show that obviously the replication in these known virus-infected cell lines or cell cultures is completely insufficient.

Furthermore it is known from the publications of Yoo et al. (1995, *Journal of Virology*, 69, 32 - 38) and of Dash et al., (1997, *American Journal of Pathology*, 151, 363 - 373) that hepatoma cell lines can be transfected with synthetic HCV-RNA, which are obtained through in vitro transcription of the cloned HCV genome. In both publications the authors started from the basic idea that the viral HCV genome is a plus-strand RNA functioning directly as mRNA after being transfected into the cell, permitting the synthesis of viral proteins in the course of the translation process, and so new HCV particles are (could be) formed. This viral replication, which means these newly formed HCV viruses and their RNA, have been detected through RT-PCR. However the published results of the RT-PCR carried out indicate, that the HCV replication in the described HCV transfected hepatoma cells is not particularly efficient and is not sufficient to measure the quality, let alone the quantity of the fluctuations in the replication rate after an targeted action with prospective antiviral treatments. Furthermore it is prior art (Yanagi et al., Proc. Natl. Acad. Sci. USA, 96, 2291-95, 1999), that the highly conserved 3' NTR is essential for the virus replication. This knowledge strictly contradicts the statements of Yoo et al. and Dash et al., who used for their experiments only HCV genomes with shorter 3' NTRs since they did not know the authentic 3' end of the HCV genome.

The object of the present invention is to provide a HCV cell culture system, where the viral RNA self-replicates in the transfected cells with such a high efficiency that the quality and quantity of the fluctuations in the replication rate can be measured with common methodologies usually found in the laboratory after a targeted action with virus and prospective HCV specific antivirals in particular.

The solution to this problem is to provide a cell culture system of the prior mentioned type, where the eukaryotic

cells are human cells, in particular hepatoma cells, which are preferably derived from a normal hepatoma cell line, but can also be obtained from an appropriate primary cell culture, and where the transfected HCV specific genetic material is a HCV-RNA construct, which essentially comprises the HCV specific RNA segments 5' NTR, NS3, NS4A, NS4B, NS5A, NS5B, and 3' NTR preferably in the order mentioned as well as a minimum of one marker gene for selection (selection gene).

Here and in the following "NTR" stands for "non-translated region" and is a known and familiar term or abbreviation to the relevant expert.

Here and in the following the term "HCV-RNA construct" comprises constructs, which include the complete HCV genome, as well as those, which only include a part of it, which means a HCV subgenome.

A preferred variation of the cell culture system according to the invention, which had proven to be worthwhile in practice, is lodged at the DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German collection of Microorganisms and Cell Cultures) in Braunschweig, Germany under the number DSM ACC2394 (laboratory name HuBl 9-13).

With the cell culture system according to the invention an in vitro system is provided for the first time, where HCV-RNA is self-replicated and expressed intracellularly and in a sufficient amount, so that the quantity of the amounts of HCV- RNA as well as the HCV specific proteins can be determined with conventional and reliably precise biochemical measuring methods. This means an almost authentic cell-based HCV replication system is available for the first time, which is urgently needed for the development and testing of antiviral drugs. This test system provides the possibility of identifying potential

targets for an effective HCV specific therapy and developing and evaluating HCV specific chemotherapeuticals.

The invention is based on the surprising finding that efficient replication of the HCV-RNA only occurs in cells if they have been transfected with an HCV-RNA construct, which comprises at least the 5' and the 3' non-translated regions (NTR) and the non-structural proteins (NS) 3 to 5B and additionally a marker gene for selection (selection gene). The structural genes are obviously without great importance for replication, whereas efficient replication of the HCV-RNA apparently only occurs if the transfected cells are subject to permanent selection pressure, which is imparted by the marker gene for selection (selection gene) linked to the HCV-RNA. Consequently the marker gene (selection gene) seems on one hand to provoke the selection of those cells, where the HCV-RNA replicates productively, and it seems on the other hand to considerably increase the efficiency of the RNA replication.

An object of the invention is also a cell-free HCV-RNA construct, characterized in that it comprises the HCV specific RNA segments 5' NTR, NS3, NS4A, NS4B, NS5A, NS5B, and 3' NTR, preferably in the order mentioned, as well as a marker gene for selection (selection gene).

In the present context the terms 5' NTR and NS3 and NS4A and NS4B and NS5A and NS5B and 3' NTR comprise each nucleotide sequence, which is described in the state of the art as the nucleotide sequence for each functional segment of the HCV genome.

By providing a HCV-RNA construct such as this, a detailed analysis of the HCV replication, pathogenesis and evolution in cell culture is possible for the first time. The HCV specific viral RNA can specifically be created as a complete genome or subgenome in any amount, and it is

possible, to manipulate the RNA construct and consequently to examine and identify the HCV functions on a genetic level.

Because all HCV enzymes identified as a main target for a therapy at the moment, namely the NS3/4A protease, the NS3 helicase and the NS5B polymerase, are included in the HCV-RNA construct according to the invention, it can be used for all relevant analyses.

An embodiment of the HCV-RNA construct, which has proven to be worthwhile in practical use, stands out by the fact that it comprises the nucleotide sequence according to the sequence protocol SEQ ID NO:1.

Further embodiments with similar good properties for practical use are characterized in that they comprise a nucleotide sequence either according to sequence protocol SEQ ID NO:2 or according to sequence protocol SEQ ID NO:3 or according to sequence protocol SEQ ID NO:4 or according to sequence protocol SEQ ID NO:5 or according to sequence protocol SEQ ID NO:6 or according to sequence protocol SEQ ID NO:7 or according to sequence protocol SEQ ID NO:8 or according to sequence protocol SEQ ID NO:9 or according to sequence protocol SEQ ID NO:10 or according to sequence protocol SEQ ID NO:11.

It is possible to provide the HCV subgenomic construct with a 3' NTR, which has a nucleotide sequence so far unknown in the state of art, a nucleotide sequence, which has been selected from the group of nucleotide sequences (a) to (i) listed in the following:

(a) ACGGGGAGCTAAACACTCCAGGCCAATAGGCCATCCTGTTTTTTTTTTTAGCTTT
TTTTTTTTTCTTTTTTTTTGAGAGAGAGAGTCTCACTCTGTTGCCCAGACTGGAG
T

(b) ACGGGGAGCTAAACACTCCAGGCCAATAGGCCATCCTGTTTTTT TTTTTAGTCT TTTTTTTTTC TTTTTTTTGA GAGAGAGAGT CTCACTCTGT TGCCCAGACT GGAGC

(c) ACGGGGAGCTAAACACTCCAGGCCAATAGGCCATCCTGTTTTTT TTTAATCTTT TTTTTTTTCT TTTTTTTTGA GAGAGAGAGT CTCACTCTGT TGCCCAGACT GCAGC

(d) ACGGGGAGCTAAACACTCCAGGCCAATAGGCCATCCTGTTTTTT TTTTTTAGTC TTTTTTTTTT TCTTTTTTTT TGAGAGAGAG AGTCTCACTC TGTTGCCCAG ACTGGAGT

(e) ACGGGGAGCTAAACACTCCAGGCCAATAGGCCATCCTGTTTTTT TTTTTAGTCT TTTTTTTTTT TCTTTTTTTT TGAGAGAGAG AGTCTCACTC TGTTGCCCAG ACTGGAGT

(f) ACGGGGAGCTAAACACTCCAGGCCAATAGGCCATCCTGTTTTTT TTTTTAGTCT TTTTTTTTTT TCTTTTTTTT TTGAGAGAGA GAGTCTCACT CTGTTGCCCA GACTGGAGT

(g) ACGGGGAGCTAAACACTCCAGGCCAATAGGCCATCCTGTTTTTT TTTTTAGTCT TTTTTTTTTT CTTTTTTTTT GAGAGAGAGA GTCTCACTCT GTTGCCCAGA CTGGAGT

(h) ACGGGGAGCTAAACACTCCAGGCCAATAGGCCATCCTGTTTTTT TTTTTTTAAT CTTTTTTTTT TTTTTCCTTT TTTTGAGAGA GAGAGTCTCA CTCTGTTGCC CAGACTGGAG T

(i) ACGGGGAGCTAAACACTCCAGGCCAATAGGCCATCCTGTTTTTT TTTTTTAATC TTTTTTTTTT TTTTCTTTTT TTTTTGAGAG AGAGAGTCTC ACTCTGTTGC CCAGACTGGA GT

The marker gene for selection (selection gene) included in the HCV-RNA constructs according to the invention is preferably a resistance gene, in particular an antibiotic resistance gene.

This has the advantage that the cells transfected with this construct can easily be selected from the non-transfected cells by adding for example the appropriate antibiotic to the cell culture medium in the case of an antibiotic resistance gene.

In the present context 'antibiotic' means any substance, which impedes the non-transfected host cells or the cells, where the HCV-RNA is not replicating efficiently, continuing to live or grow, especially the cell poison Puromycin, Hygromycin, Zeocin, Bleomycin or Blasticidin.

A preferred marker gene for selection (selection gene) and resistance gene, which has proven to be worthwhile in practice, is the neomycin phosphotransferase gene.

An alternative for the antibiotic resistance genes is for example the thymidine kinase gene, which can be used to carry out a HAT selection.

The marker gene for selection (selection gene), the preferred resistance gene and the most preferred antibiotic resistance gene is preferably positioned in the HCV-RNA construct after the HCV 5' NTR, which means downstream from the 5' NTR and upstream from the HCV reading frame. However, an insertion in the area of the 3' NTR or another site of the HCV genome or subgenome, for example within the polyprotein, is also conceivable.

In another embodiment of the HCV-RNA construct according to the invention the marker gene for selection (selection gene), in particular an antibiotic resistance gene, is linked to the HCV-RNA or HCV genomic or subgenomic sequence via a ribozyme or a recognition site for a ribozyme.

This has the advantage, that after the selection of the cells, in which the HCV-RNA is replicating productively,

the resistance gene in the obtained cell clones can be separated from the HCV subgenomic sequence through a ribozyme-dependent cleavage, namely by activating the inserted ribozyme or in the case of a construct with a recognition site for a ribozyme, by transfecting the ribozyme into the cells (for example through the transfection of a ribozyme construct or infection with a viral expression vector, into which the appropriate ribozyme has been inserted). By this means an authentic HCV genomic construct can be obtained without a resistance gene, which can then form authentic infectious virus particles.

Another preferred embodiment of the HCV-RNA construct according to the invention is characterized in that the construct has at least one integrated reporter gene.

In the following a reporter gene means any gene, whose presence can be easily detected with, in general, simple biochemical or also histochemical methods after being transferred into a target organism, which means a gene, that encodes for a protein, which can be easily and reliably detected and quantified in small amounts with the common measuring methods in the laboratory.

This variation of the HCV-RNA construct has the advantage that the extent of the replication of this construct can be easily and quickly measured with the methods common in the laboratory using the reporter gene product.

The reporter gene is preferably a gene from the group of the luciferase genes, the CAT gene (chloramphenicol acetyl transferase gene), the lacZ gene (beta galactosidase gene), the GFP gene (green fluorescence protein gene), the GUS gene (glucuronidase gene) or the SEAP gene (secreted alkaline phosphatase gene). This reporter gene and its products, namely the relevant reporter proteins, can be

detected for example using fluorescence, chemiluminescence, colorimetric measurements or by means of immunological methods (for example ELISA).

A surrogate marker gene can also be considered as a reporter gene. In this context it includes those genes, which encode for cellular proteins, nucleic acids or generally for those functions, which are subject to variation depending on the replication of the virus, and which consequently are either suppressed or activated in the cells, in which the HCV or the HCV-RNA construct multiplies. This means, the suppression or activation of this function is a surrogate marker for the replication of the virus or the replication of the HCV-RNA construct.

The positions of the reporter genes and the marker gene for selection (selection gene) can be selected in such a way, that a fusion protein made from both genetic products will be expressed. This has the advantage that these two genes can be arranged in such a way in the HCV-RNA construct that their two expressed proteins are fused via a recognition sequence for a protease (for example ubiquitin) or via a self-cleaving peptide (for example the 2A protein of the Picornaviruses) at first and will be separated proteolytically later.

These two positions might as well lie apart from each other in such way, that both genetic products are separately expressed (for example in the order: marker or resistance gene – internal ribosome binding site – reporter gene).

In the case of the reporter gene one embodiment has proven to be particularly worthwhile, where the reporter gene is cloned into the open reading frame of the HCV genome or subgenome in such a way that it will only be transferred to an active form after proteolytic processing.

The cell culture system according to the invention can be used for various purposes in each of its embodiments. These comprise:

- The detection of antiviral substances. This can include for example: organic compounds, which interfere directly or indirectly with viral growth (for example inhibitors of the viral proteases, the NS3 helicase, the NS5B RNA-dependent RNA polymerase), antisense oligonucleotides, which will hybridize to any target sequence in the HCV-RNA construct (for example the 5' NTR) and will have an direct or indirect influence on the virus growth for example due to a reduction of translation of the HCV polyprotein or ribozymes, which cleave any HCV-RNA sequence and consequently impair virus replication.

- The evaluation of any type of antiviral substances in the cell culture. These substances can be detected on the isolated purified enzyme for example with 'rational drug design' or 'high-throughput screening'. Evaluation means mainly the determination of the inhibitory features of the respective substance as well as its mode of action.

- The identification of new targets of viral or cellular origin for a HCV specific antiviral therapy. If for example a cellular protein is essential for viral replication, the viral replication can also be influenced by inhibiting this cellular protein. The system according to the invention also enables the detection of these auxiliary factors.

- The determination of drug resistance. It can be assumed that resistance to therapy occurs due to the high mutation rate of the HCV genome. This resistance, which is very important for the clinical approval of a substance, can be detected with the cell culture system

according to the invention. Cell lines, in which the HCV-RNA construct or the HCV genome or subgenome replicates, are incubated with increasing concentrations of the relevant substance and the replication of the viral RNA is either determined by means of an introduced reporter gene or through the qualitative or quantitative detection of the viral nucleic acids or proteins. Resistance is given if no or a reduced inhibition of the replication can be observed with the normal concentration of the active substance. The nucleotide and amino acid replacements responsible for the therapy resistance can be determined by recloning the HCV-RNA (for example by the means of RT-PCR) and sequence analysis. By cloning the relevant replacement/s into the original construct its causality for the resistance to therapy can be proven.

- The production of authentic virus proteins (antigens) for the development and/or evaluation of diagnostics. The cell culture system according to the invention also allows the expression of HCV antigens in cell cultures. In principle these antigens can be used as the basis for diagnostic detection methods.

- The production of HCV viruses and virus-like particles, in particular for the development or production of therapeutics and vaccines as well as for diagnostic purposes. Especially cell culture adapted complete HCV genomes, which could be produced by using the cell cultur system according to the invention, are able to replicate in cell culture with high efficiency. These genomes have the complete functions of HCV and in consequence they are able to produce infectious viruses.

The HCV-RNA construct according to the invention by itself can also be used for various purposes in all its embodiments. This includes first of all:

- The construction of attenuated hepatitis C viruses or HCV-like particles and their production in cell cultures:

  Attenuated HCV or HCV-like particles can be created by accidental or purposefully introduced mutations, such as point mutations, deletions or insertions, which means viruses or virus-like particles with complete ability to replicate, but reduced or missing pathogenicity. These attenuated HCV or HCV-like particles can be used in particular as vaccine.

- The construction of HCV-RNA constructs with integrated foreign genes, used for example as liver cell specific vector in gene therapy. Due to the distinctive liver cell tropism of the HCV and the possibility of replacing parts of the genome by heterologous sequences, HCV-RNA constructs can be produced, where for example the structural proteins can be replaced by a therapeutically effective gene. The HCV-RNA construct obtained in this way is introduced into cells preferably by means of transfection, which express the missing HCV functions, for example the structural proteins, in a constitutive or inducible way. Virus particles, carrying the HCV-RNA construct, can be created by means of this method known to the expert under the term 'transcomplementation'. The particles obtained can preferably be used for the infection of liver cells. Within these the therapeutically effective foreign gene will be expressed and will consequently develop its therapeutic effect.

- The detection of permissive cells, which means cells, in which a productive virus growth occurs. For this purpose either one of the HCV-RNA genomic constructs previously mentioned, which is able to form complete infectious viruses, or one of the HCV subgenomes previously

mentioned, which according to the previously mentioned example will be transfected in a cell line first, which expresses the missing functions in a constitutive or inducible way, is used. In each case virus particles are created, which carry a resistance and/or reporter gene apart from the HCV sequence. In order to detect cells, where the HCV is able to replicate, these cells are infected with viruses generated in this way and subject to an antibiotic selection or they are examined depending on the HCV-RNA construct by means of determining the presence of the expression of the reporter gene. Because an antibiotic resistance or reporter gene expression can only be established, when HCV-RNA construct replicates, the cells detected in this way must be permissive. Almost any cell line or primary cell culture can be tested in regard to the permissivity and detected in this way.

The cell culture system according to the invention also permits targeted discovery of HCV-RNA constructs for which there is an increase in the efficiency of replication due to mutations. This occurs either by chance, in the context of HCV-RNA replication, or by targeted introduction into the construct. These mutations, leading to a change in the replication of the HCV-RNA construct, are known to experts as adaptive mutations. The invention therefore also includes a method for obtaining cell culture adapted mutants of a HCV-RNA construct according to the invention following the above description, in which the mutants have increased replication efficiency compared to the original HCV-RNA construct. It further includes a method for the production of mutants of a HCV-RNA full-length genome or of a HCV-RNA subgenome or of any HCV-RNA construct with increased replication efficiency compared to the original HCV-RNA full-length genome or subgenome or HCV-RNA construct, as well as cell culture adapted mutants of HCV-

RNA constructs, HCV-RNA full-length genomes and HCV subgenomes with increased replication efficiency compared to the original constructs, subgenomes or full-length genomes.

The method according to the invention for the production of cell culture adapted mutants of a HCV-RNA construct according to the invention, in which the mutants have increased replication efficiency compared to the HCV-RNA construct, is characterised in that a cell culture system according to claim 1, in which the transfected HCV specific genetic material is a HCV-RNA construct with a selection gene according to claims 4 to 19, is cultivated on/in the selection medium corresponding to the selection gene, that the cultivated cell clones are collected and that the HCV-RNA construct is isolated from these cell clones.

In an advantageous extension of this production method, the isolated HCV-RNA constructs are passaged at least one more time, that is they are transfected in cells of a cell culture system according to claim 1, the thus obtained cell culture system according to claim 1, in which the transfected HCV specific genetic material is the isolated HCV-RNA construct with a selection gene, is cultivated on/in the selection medium corresponding to the selection gene, the cultivated cell clones are collected and the HCV-RNA constructs are thus isolated.

Using this process variation, the quantity of adaptive mutations and hence the degree of replication efficiency in the relevant HCV-RNA constructs can be increased even further.

The method according to the invention for the production of mutants of a HCV-RNA full-length genome or of a HCV-RNA subgenome or of any HCV-RNA construct with increased replication efficiency compared to the original HCV-RNA

full-length genome or subgenome or HCV-RNA construct, has the following features. Using one of the two production methods presented above, a cell culture adapted mutant of a HCV-RNA construct is produced, isolated from the cells, cloned using state of the art known methods and sequenced. By comparing with the nucleotide and amino acid sequence of the original HCV-RNA construct, the type, number and position of the mutations is determined. These mutations are then introduced into an (isolated) HCV subgenome or full-length genome or any HCV-RNA construct, either by site-directed mutagenesis, or by exchange of DNA fragments containing the relevant mutations.

A test can be carried out to determine or verify which mutations actually are responsible for an alteration of replication efficiency, particularly an increase in replication. In this test the corresponding nucleotide and/or amino acid changes are introduced into the original HCV-RNA construct and the modified construct is then transfected in cell culture. If the introduced mutation actually leads to an increase in replication, then for a HCV-RNA construct with a selectable marker gene, the number of resistant cell clones in the artificially mutated construct should be noticeably higher compared to the untreated construct.

In the case of a construct with a reporter gene, the activity or quantity of the reporter should be noticeably higher for the artificially mutated construct compared to the untreated one.

The cell culture adapted HCV-RNA constructs with high replication efficiency according to the invention are characterized in that, through nucleotide or amino acid exchanges, they are derivable from a HCV-RNA construct according to one of the claims 4 to 19, and that they are

obtainable using one of the two production processes presented above.

These cell culture adapted HCV-RNA constructs can be used to produce any HCV-RNA constructs or HCV full-length or subgenomes with increased replication efficiency. Both constructs with a selectable resistance gene and constructs without one or with a non-selectable reporter gene (e.g. luciferase) can be produced in this way, since replication of cell culture adapted HCV-RNA constructs can also be demonstrated in non-selected cells due to their high replication efficiency.

The cell culture adapted mutants of a HCV-RNA construct or HCV-RNA full-length genome or HCV subgenome with high replication efficiency compared to the original HCV-RNA construct or the original HCV full-length genome are characterized in that they are obtainable by a method in which the type and number of mutations in a cell culture adapted HCV-RNA construct are determined through sequence analysis and sequence comparison and these mutations are introduced into a HCV-RNA construct, particularly a HCV-RNA construct according to one of the claims 4 to 19, or into an (isolated) HCV-RNA full-length genome, either by site-directed mutagenesis, or by exchange of DNA fragments containing the relevant mutations.

A group of preferred HCV-RNA constructs, HCV full-length genomes and HCV subgenomes with high and very high replication efficiency, which are consequently highly suitable for practical use is characterised in that it contains one, several or all of the amino acid or nucleic acid exchanges listed in table 3 and/or one or several of the following amino acid exchanges: 1283 arg -> gly , 1383 glu -> ala , 1577 lys -> arg , 1609 lys -> glu , 1936 pro -> ser , 2163 glu -> gly , 2330 lys -> glu , 2442 ile

-> val. (The numbers refer to the amino acid positions of the polyprotein of the HCV isolate con1, see Table 1).

Special features of the nucleotid sequences according to the sequence listings:

SEQ ID-NO: 1

Name: I389/Core-3'/wt

Composition (Nucleotide positions):

1. 1-341: HCV 5' non-translated region
2. 342-1193: HCV Core Protein-Neomycin Phosphotransferase fusion protein; selectable Marker
3. 1202-1812: internal ribosome entry site from encephalomyokarditis virus; directs translation of the downstream located HCV open reading frame
4. 1813-10842: HCV Polyprotein from Core up to nonstructural protein 5B
    5. 1813-2385: HCV Core Protein; structural protein
    6. 2386-2961: envelope protein 1 (E1); structural protein
    7. 2962-4050: envelope protein 2 (E2); structural protein
    8. 4051-4239: Protein p7
    9. 4240-4890: nonstructural protein 2 (NS2); HCV NS2-3 Protease
    10. 4891-6783: nonstructural protein 3 (NS3); HCV NS3 Protease/Helicase
    11. 6784-6945: nonstructural protein 4A (NS4A); NS3 Protease cofactor
    12. 6946-7728: nonstructural protein 4B (NS4B)
    13. 7729-9069: nonstructural protein 5A (NS5A)
    14. 9070-10842: nonstructural protein 5B (NS5B); RNA-dependent RNA-polymerase
5. 10846-11076: HCV 3' non-translated region

SEQ ID-NO: 2

Name: I337/NS2-3'/wt

Composition (Nucleotide positions):

1.1-341: HCV 5' non-translated region

2.342-1181: HCV Core Protein-Neomycin Phosphotransferase fusion protein; selectable Marker

3.1190-1800: internal ribosome entry site from encephalomyokarditis virus; directs translation of the downstream located HCV open reading frame

4.1801-8403: HCV Polyprotein from nonstructural protein 2 up to nonstructural protein 5B

5. 1801-2451: nonstructural protein 2 (NS2); HCV NS2-3 Protease

6. 2452-4344: nonstructural protein 3 (NS3); HCV NS3 Protease/Helicase

7. 4345-4506: nonstructural protein 4A (NS4A); NS3 Protease cofactor

8. 4507-5289: nonstructural protein 4B (NS4B)

9. 5290-6630: nonstructural protein 5A (NS5A)

10. 6631-8403: nonstructural protein 5B (NS5B); RNA-dependent RNA-polymerase

11. 8407-8637: HCV 3' non-translated region

SEQ ID-NO: 3

Name: I389/NS3-3'/wt

Composition (Nucleotide positions):

1.1-341: HCV 5' non-translated region

2.342-1193: HCV Core Protein-Neomycin Phosphotransferase fusion protein; selectable Marker

3.1202-1812: internal ribosome entry site from encephalomyokarditis virus; directs translation of the downstream located HCV open reading frame

4.1813-7767: HCV Polyprotein from nonstructural protein 3 up to nonstructural protein 5B

5. 1813-3708: nonstructural protein 3 (NS3); HCV NS3 Protease/Helicase
6. 3709-3870: Nonstructural protein 4A (NS4A); NS3 Protease Cofactor
7. 3871-4653: Nonstructural protein 4B (NS4B)
8. 4654-5994: Nonstructural protein 5A (NS5A)
9. 5995-7767: Nonstructural protein 5B (NS5B); RNA-dependent RNA-Polymerase
10. 7771-8001: HCV 3' non-translated Region

SEQ ID-NO: 4

Name: I337/NS3-3'/wt

Composition (Nucleotide positions):

1.1-341: HCV 5' non-translated region

2.342-1181: HCV Core Protein-Neomycin Phosphotransferase fusion protein; selectable Marker

3.1190-1800: internal ribosome entry site from encephalomyokarditis virus; directs translation of the downstream located HCV open reading frame

4.1801-7758: HCV Polyprotein from Nonstructural protein 3 up to Nonstructural protein 5B

5. 1801-3696: Nonstructural protein 3 (NS3); HCV NS3 Protease/Helicase
6. 3697-3858: Nonstructural protein 4A (NS4A); NS3 Protease Cofactor
7. 3859-4641: Nonstructural protein 4B (NS4B)
8. 4642-5982: Nonstructural protein 5A (NS5A)
9. 5983-7755: Nonstructural protein 5B (NS5B); RNA-dependent RNA-Polymerase
10. 7759-7989: HCV 3' non-translated Region

SEQ ID-NO: 5

Name: I389/NS2-3'/wt

Composition (Nucleotide positions):

1.1-341: HCV 5' non-translated region
2.342-1193: HCV Core Protein-Neomycin Phosphotransferase fusion protein; selectable Marker
3.1202-1812: internal ribosome entry site from encephalomyokarditis virus; directs translation of the downstream located HCV open reading frame
4.1813-8418: HCV Polyprotein from Nonstructural protein 2 up to Nonstructural protein 5B
5. 1813-2463: Nonstructural protein 2 (NS2); HCV NS2-3 Protease
6. 2464-4356: Nonstructural protein 3 (NS3); HCV NS3 Protease/Helicase
7. 4357-4518: Nonstructural protein 4A (NS4A); NS3 Protease Cofactor
8. 4519-5301: Nonstructural protein 4B (NS4B)
9. 5302-6642: Nonstructural protein 5A (NS5A)
10. 6643-8415: Nonstructural protein 5B (NS5B); RNA-dependent RNA-Polymerase
11. 8419-8649: HCV 3' non-translated Region

SEQ ID-NO: 6

Name: I389/NS3-3'/9-13F
Composition (Nucleotide positions):
1.1-341: HCV 5' non-translated region
2.342-1193: HCV Core Protein-Neomycin Phosphotransferase fusion protein; selectable Marker
3.1202-1812: internal ribosome entry site from encephalomyokarditis virus; directs translation of the downstream located HCV open reading frame
4.1813-7767: HCV Polyprotein from Nonstructural protein 3 up to Nonstructural protein 5B of the cell culture-adapted mutant 9-13F
5. 1813-3708: Nonstructural protein 3 (NS3); HCV NS3 Protease/Helicase
6. 3709-3870: Nonstructural protein 4A (NS4A); NS3

Protease Cofactor

7. 3871-4653: Nonstructural protein 4B (NS4B)
8. 4654-5994: Nonstructural protein 5A (NS5A)
9. 5995-7767: Nonstructural protein 5B (NS5B); RNA-dependent RNA-Polymerase

7771-8001: HCV 3' non-translated Region

SEQ ID-NO: 7

Name: I389/Core-3'/9-13F

Composition (Nucleotide positions):

1.1-341: HCV 5' non-translated region

2.342-1193: HCV Core Protein-Neomycin Phosphotransferase fusion protein; selectable Marker

3.1202-1812: internal ribosome entry site from encephalomyokarditis virus; directs translation of the downstream located HCV open reading frame

4.1813-10842: HCV Polyprotein from Core up to Nonstructural protein 5B of the cell culture-adapted mutant 9-13F

5. 1813-2385: HCV Core Protein; structural protein
6. 2386-2961: envelope protein 1 (E1); structural protein
7. 2962-4050: envelope protein 2 (E2); structural protein
8. 4051-4239: Protein p7
9. 4240-4890: Nonstructural protein 2 (NS2); HCV NS2-3 Protease
10. 4891-6783: Nonstructural protein 3 (NS3); HCV NS3 Protease/Helicase
11. 6784-6945: Nonstructural protein 4A (NS4A); NS3 Protease Cofactor
12. 6946-7728: Nonstructural protein 4B (NS4B)
13. 7729-9069: Nonstructural protein 5A (NS5A)
14. 9070-10842: Nonstructural protein 5B (NS5B); RNA-dependent RNA-Polymerase
15. 10846-11076: HCV 3' non-translated Region

SEQ ID-NO: 8

Name: I389/NS3-3'/5.1

Composition (Nucleotide positions):

1.1-341: HCV 5' non-translated region

2.342-1193: HCV Core Protein-Neomycin Phosphotransferase fusion protein; selectable Marker

3.1202-1812: internal ribosome entry site from encephalomyokarditis virus; directs translation of the downstream located HCV open reading frame

4.1813-7767: HCV Polyprotein from Nonstructural protein 3 up to Nonstructural protein 5B of the cell culture-adapted mutant 5.1

5. 1813-3708: Nonstructural protein 3 (NS3); HCV NS3 Protease/Helicase

6. 3709-3870: Nonstructural protein 4A (NS4A); NS3 Protease Cofactor

7. 3871-4653: Nonstructural protein 4B (NS4B)

8. 4654-5994: Nonstructural protein 5A (NS5A)

9. 5995-7767: Nonstructural protein 5B (NS5B); RNA-dependent RNA-Polymerase

7771-8001: HCV 3' non-translated Region

SEQ ID-NO: 9

Name: I389/Core-3'/5.1

Composition (Nucleotide positions):

1.1-341: HCV 5' non-translated region

2.342-1193: HCV Core Protein-Neomycin Phosphotransferase fusion protein; selectable Marker

3.1202-1812: internal ribosome entry site from encephalomyokarditis virus; directs translation of the downstream located HCV open reading frame

4.1813-10842: HCV Polyprotein from Core up to Nonstructural protein 5B of the cell culture-adapted mutant 5.1

5. 1813-2385: HCV Core Protein; structural protein

6. 2386-2961: envelope protein 1 (E1); structural protein
7. 2962-4050: envelope protein 2 (E2); structural protein
8. 4051-4239: Protein p7
9. 4240-4890: Nonstructural protein 2 (NS2); HCV NS2-3 Protease
10. 4891-6783: Nonstructural protein 3 (NS3); HCV NS3 Protease/Helicase
11. 6784-6945: Nonstructural protein 4A (NS4A); NS3 Protease Cofactor
12. 6946-7728: Nonstructural protein 4B (NS4B)
13. 7729-9069: Nonstructural protein 5A (NS5A)
14. 9070-10842: Nonstructural protein 5B (NS5B); RNA-dependent RNA-Polymerase
15. 10846-11076: HCV 3' non-translated Region

SEQ ID-NO: 10

Name: I389/NS3-3'/19

Composition (Nucleotide positions):

1. 1-341: HCV 5' non-translated region
2. 342-1193: HCV Core Protein-Neomycin Phosphotransferase fusion protein; selectable Marker
3. 1202-1812: internal ribosome entry site from encephalomyokarditis virus; directs translation of the downstream located HCV open reading frame
4. 1813-7767: HCV Polyprotein from Nonstructural protein 3 up to Nonstructural protein 5B of the cell culture-adapted mutant 19
5. 1813-3708: Nonstructural protein 3 (NS3); HCV NS3 Protease/Helicase
6. 3709-3870: Nonstructural protein 4A (NS4A); NS3 Protease Cofactor
7. 3871-4653: Nonstructural protein 4B (NS4B)
8. 4654-5994: Nonstructural protein 5A (NS5A)

9. 5995-7767: Nonstructural protein 5B (NS5B); RNA-dependent RNA-Polymerase

7771-8001: HCV 3' non-translated Region

SEQ ID-NO: 11

Name: I389/Core-3'/19

Composition (Nucleotide positions):

1.1-341: HCV 5' non-translated region

2.342-1193: HCV Core Protein-Neomycin Phosphotransferase fusion protein; selectable Marker

3.1202-1812: internal ribosome entry site from encephalomyokarditis virus; directs translation of the downstream located HCV open reading frame

4.1813-10842: HCV Polyprotein from Core up to Nonstructural protein 5B of the cell culture-adpated mutant 19

5. 1813-2385: HCV Core Protein; structural protein

6. 2386-2961: envelope protein 1 (E1); structural protein

7. 2962-4050: envelope protein 2 (E2); structural protein

8. 4051-4239: Protein p7

9. 4240-4890: Nonstructural protein 2 (NS2); HCV NS2-3 Protease

10.4891-6783: Nonstructural protein 3 (NS3); HCV NS3 Protease/Helicase

11.6784-6945: Nonstructural protein 4A (NS4A); NS3 Protease Cofactor

12.6946-7728: Nonstructural protein 4B (NS4B)

13.7729-9069: Nonstructural protein 5A (NS5A)

14.9070-10842: Nonstructural protein 5B (NS5B); RNA-dependent RNA-Polymerase

15.10846-11076: HCV 3' non-translated Region

The invention is described in detail in the following by way of examples of embodiments and respective diagrams. These are as follows

Fig. 1 A: The structure of a HCV-RNA construct according to the invention

On the top a diagram of the structure of the complete parental HCV genome is given with the positions of the genes for the cleavage products core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B within the polyprotein, and the 5' and 3' non-translated regions (5' NTR and 3' NTR) - shown as a thick horizontal line -, and with the two positions selected for the creation of the subgenomic constructs, namely the position of the 'GDD catalytic domain' of the NS5B RNA polymerase (GDD) and the position of the 3' end of the HCV-IRES (nucleotide positions 1 to 377 and 1 to 389) - drawn above the diagram of the genome. The numbers below the diagram of the genome indicate the respective nucleotide positions.

Diagrams of the structure of two modified HCV-RNA constructs (subgenome) according to the invention are shown below, consisting of the 5' HCV-IRES, the neomycin phosphotransferase gene (Neo^R), the EMCV-IRES (E-I) and the HCV sequences of NS2 or NS3 up to the authentic 3' end. The position of the 10-amino acid deletion comprising the NS5B polymerase GDD motive is marked with a triangle (Δ).

Fig. 1 B: The result of a denaturing formaldehyde-agarose gel electrophoresis for the detection of replicating plus-strand RNA in transfected subpassaged Huh 7 cell clones.

The positions of HCV specific RNAs (arrows) and the 28S rRNA are specified to the right of lane 12, the size (number of nucleotides) of the RNA marker (M) is specified to the left of lane 1.

Fig. 1 C: The result of a PCR test with subsequent Southern Blot to demonstrate the absence of integrated replicon DNA in most of the selected cell clones.

The lanes 1 and 2 show the positive control, lane 13 the negative control. The figures to the left of lane 1 indicate the size of the nucleotide marker molecules.

Fig. 2 A: The result of a PCR test with subsequent Southern Blot for the detection of the sensitive exclusion of integrated replicon DNA (plasmid molecule I_{377}/NS3-3'/wt) in a cell clone containing a HCV-RNA construct (9-13).

The lanes 7 to 11 represent the result of a titration of DNA molecules of the construct I_{377}/NS3-3'/wt without addition of total DNA of the cell clone 9-13, and the lanes 2 - 6 represent the result obtained with the same plasmid molecules with the addition of 1 µg 9-13 DNA each prior to the PCR (for the purpose of excluding an inhibitor of the PCR in the DNA preparation). Lane 13 represents the negative control (PCR without DNA template). Lane 1 shows the result, which was achieved with one µg total DNA of the cell clone 9-13.

Fig. 2 B: The result of a Northern Blot test to quantify the amounts of HCV plus- and minus-strand RNA.

The arrows mark the positions of replicon RNA.

The "plus" and "minus" details indicate the positive (plus) and negative (minus) polarity of the RNA controls, which have been applied to the gel. "Minus-strand" and "Plus-strand" indicate the specificity of the radioactive RNA probes.

Fig. 2 C: The result of a formaldehyde-agarose gel electrophoresis after radioactive labeling of the intracellular replicated HCV-RNA to demonstrate the resistance of HCV-RNA replication to dactinomycin.

Fig. 3 A: The detection of HCV specific antigens in the selected cell clones by means of immunoprecipitation after metabolic radioactive labeling.

The lanes 7 - 9 represent authentic size marker (which have been obtained in Huh 7 cells after the transient expression of a HCV-RNA construct); identified HCV proteins are labeled on the left edge of lane 1, the molecular weights (in Kilodalton) are specified to the right of lane 9.

Fig. 3 B: Results of an immunofluorescence test to establish the subcellular localisation of HCV antigens.

Fig. 4 : Diagram of the structure of a selectable HCV-RNA construct according to the invention (complete genome) consisting of the 5' HCV-IRES, the neomycin phosphotransferase gene (NeoR), a heterologous IRES element, for example from the encephalomyocarditis virus (E-I), the complete HCV open reading frame and the authentic 3' NTR.

Fig. 5: Diagram of the structure of HCV-RNA constructs with inserted antibiotic resistance gene **(A)** within the nucleotide sequence encoding the polyprotein (monocistronic RNA construct), and **(B)** within the 3' NTR (bicistronic RNA construct).

Fig. 6: Diagram of the structure of HCV-RNA constructs with inserted reporter genes **(A)** as part of a HCV replicon from NS3 to NS5B, - in the end the reporter protein is cleaved by viral or cellular proteases out of the polyprotein or the marker gene for selection (selection gene) or the resistance gene are transfected into the cells through co-transfection -, **(B)** as part of a fusion gene composed of a resistance and reporter gene (for example for the neomycin phosphotransferase and green fluorescent protein), **(C)** as part of a replicon composed of a resistance and reporter gene (for example the neomycin phosphotransferase and green fluorescent protein) bound via a nucleotide sequence, which encodes for an amino acid sequence (area with hatches), which can be cleaved by a protease or has a self-cleaving (autocatalytic) activity, **(D)** as independent gene (in this case the green fluorescent protein), which is expressed from its own internal ribosome binding site (IRES); - the resistance gene (in this case the neomycin phosphotransferase gene) is also expressed from its own internal ribosome binding site (IRES) (polycistronic construct).

Fig. 7: Diagram of the structure of a HCV-RNA construct, where the resistance gene is linked to the HCV-

RNA sequence via a ribozyme or a recognition site for a ribozyme.

The thick lines illustrate the HCV 5' and 3' NTRs, E-I is a heterologous internal ribosome binding site, which is required for the expression of the resistance gene, and the grey square illustrates the ribozyme or a recognition site for a ribozyme.

Fig. 8: Diagram of the structure of a HCV-RNA construct with resistance gene and integrated foreign gene.

Fig. 9: Method for comparing the specific infectivity (expressed as number of cell colonies obtained) of total RNA against in vitro transcripts. HCV-RNA is generated by in vitro transcription of a corresponding RNA construct and quantified by measurement of the optical density at 260 nm (OD 260 nm). A defined number of these molecules is mixed with a specified amount of total RNA from naive Huh-7 cells and this mixture is transfected into naive Huh-7 cells with the aid of electroporation. At the same time the total RNA of a cell clone, produced by the method described in Figure 1, is isolated using a known state of the art method and the amount of HCV-RNA contained therein is determined by means of Northern Blot using a HCV specific RNA probe and subsequent quantification via phosphoimaging. A defined amount of this total RNA is analogously transfected in naïve Huh-7 cells. These cells from both the cultures are then subjected to a G418 selection and the number of colonies created is determined by counting after fixing and staining with Coomassie Brilliant Blue. For the determination of transfection efficiency 1µg of a

plasmid allowing the expression of luciferase is added to each transfection culture. An aliquot of the transfected cells is collected after 24 hours and the luciferase activity determined in the respective cell lysates. The number of colonies is always normed to the luciferase expression.

Fig. 10: Sequence analysis of the 9-13 clone. Total RNA of the 9-13 cell clone, resulting from transfection of HCV-RNA construct I377/NS3-3', was isolated using a known state of the art method and the HCV-RNA construct amplified from nucleotide position 59 to 9386 with the aid of 'long-distance RT-PCR' using primer S59 and A9413. The PCR fragments were cloned and 11 clones (9-13 A - K) completely sequenced. Clones D and I, E and G as well as H and J turned out to be identical, respectively. The positions of the amino acid differences in the NS3-5B region between the recloned HCV-RNAs and the parental construct are marked with a thick vertical mark for each clone. Each clone was digested with restriction enzyme *Sfi*I and the respective fragment inserted in the parental construct. These clones were each transfected in Huh-7 cells and the cells subjected to selection as described in Figure 1. The number of cell clones obtained with each construct is noted next to the respective construct on the right.

Fig.11 A: Principle of determination of replication with the aid of a reporter gene. In the upper part of the figure, the HCV-DNA construct I_{389}/Luc/NS3-3' is shown. It consists of the HCV 5' NTR (nucleotide positions 1-389), the luciferase gene (*luc*), the IRES of the encephalomyocarditis virus, the HCV NS3-5B and the 3' NTR. The

position of the active centre of the NS5B RNA polymerase, into which a deactivating amino acid exchange was introduced, is indicated by 'GND'. The plasmids, which code for the HCV-RNA construct which is able to replicate or is defective, are digested with the restriction enzyme *ScaI* and added to an in vitro transcription reaction with T7 RNA polymerase. After removal of the template DNA, the respective HCV-RNA constructs were transfected in naive Huh-7 cells by means of electroporation and the latter collected at regular intervals.

Fig.11 B: Comparison of luciferase activity in cells transfected with the parental HCV-RNA construct I_{389}/Luc/NS3-3'/wt (wt) or the following variants: inactive RNA (318 DN), variants 9-13F or variant 5.1. The cells were collected 6 (not shown), 24, 48, 72, 96, 120, 144 and 168 hours after transfection and luciferase activities determined by luminometric measurement.

Fig. 12: Selectable HCV full-length genomes (constructs I_{389}/core 3'/5.1 and I_{389}/core 3'/9-13F).
(A) Diagram of the full-length construct. The region between both indicated recognition sites for the restriction enzyme *SfiI* corresponds to the sequences of the highly adapted RNA variants 5.1. or 9-13F.
(B) Number of colonies which were obtained after transfection of 0.1 µg in vitro transcribed RNA of the construct I_{389}/core-3'/5.1. described under A into HUH7-cells. The result of a representative experiment is given.
(C) Demonstration of autonomously replicating HCV full-length RNAs in G418 resistant cell clones which were obtained after transfection of the

corresponding in vitro transcript. The illustration shows the autoradiogram of a Northern Blot, which was hybridised with a probe against the *neo*-resistance gene and the HCV 5' NTR. The controls shown in lanes 1 and 2 each correspond to 10^8 molecules of the indicated in vitro transcripts, mixed with total RNA from naive Huh-7 cells. The negative control contains only total RNA from naive Huh-7 cells (lane 3). Lanes 4-9 contain 3-10 µg total RNA from G418 resistant cell clones, which were obtained after transfection by in vitro transcribed I_{389}/core 3'/5.1 RNA or I_{389}/core 3'/9-13F RNA. The G418 concentration used for the selection is indicated in each case. Five of the cell clones shown contain the highly adapted RNA variant 5.1 (lanes 4-8), one contains the adapted RNA variant 9-13F (lane 9).

Fig. 13: HCV-RNA constructs with a reporter gene. (A) Bicistronic HCV-RNA constructs. The reporter gene is translated with the aid of a separate IRES. (B) Monocistronic HCV-RNA constructs. The reporter gene product is expressed as fusion protein with a HCV protein. Both portions are linked via a recognition sequence for a viral or cellular protease, which permits a proteolytic separation of the two fused protein portions. In the example shown the reporter gene product and respective HCV protein was fused through a recognition sequence for ubiquitin (Ub).

Fig. 14: Tricistronic full-length HCV-RNA construct, that in addition to the resistance gene possesses an inserted foreign gene (ther. gene).

Fig. 15: Monocistronic HCV-RNA constructs, for which the resistance gene product is expressed as a fusion protein with HCV portion. The resistance gene (RG) is either active as a fusion protein or it is fused with the HCV portion via a proteolytically cleavable sequence in such a way that the resistance gene product is split from the HCV portion by a cellular or viral protease. In the example shown the resistance gene was fused with the respective HCV portion through the sequence coding for ubiquitin (Ub).

Example 1: Production of HCV-RNA constructs

(A) Synthesis and cloning of a complete HCV consensus genome by means of RT-PCR

The HCV genome, which means the HCV-RNA, was isolated from the liver of a chronically infected patient as described in the following:

The total RNA was isolated from approx. 100 mg of liver according to the method described by Chomczynski and Sacci (1987, Anal. Biochem. 162, 156). Using 1 µg of this isolated RNA a reverse transcription was carried out with the primers A6103 (GCTATCAGCCGGTTCATCCACTGC) or A9413 (CAGGATGGCCTATTGG CCTGGAG) and the expand reverse transcriptase system (Boehringer Mannheim, Germany) according to the manufacturer's recommendations. A polymerase chain reaction (PCR) was carried out with the products of this reverse transcription using the expand long template system (Boehringer Mannheim, Germany), in which the buffer containing 2 % of dimethyl sulfoxide was used. After one hour at 42 °C, 1/8 of the reaction mixture was used for a first PCR with primers A6103 and S59

(TGTCTTCACGCAGAAAGCGTCTAG) or A9413 and S4542 (GATGAGCT CGCCGCGAAGCTGTCC). After 40 cycles, 1/10 of this reaction mixture was used for a second PCR with primers S59 and A4919 (AGCACAGCCCGCGTCATAGCACTCG) or S4542 and A9386 (TTAGCTCCCCG TTCATCGGTTGG. After 30 cycles the PCR products were purified by means of preparative agarose gel electrophoresis and the eluted fragments were ligated into the vector pCR2.1 (Invitrogen) or pBSK II (Stratagene). Four clones of each fragment were analyzed and sequenced, and a consensus sequence was established. For this purpose the DNA sequences were compared to each other. The positions, where the sequence of one fragment was different from the others, were considered as undesired mutations. In the case of ambiguities of the sequence, shorter overlapping PCR fragments of the respective region were amplified and several clones sequenced. By this means several potential mutations could be identified in each fragment and consequently an isolate specific consensus sequence could be established. This established consensus sequence or genome belongs to the worldwide spread genotype 1b. The non-translated region at the 3' end (=3' NTR) was obtained by means of a conventional PCR, whereby an antisense primer, which covers the last 24 nucleotides of the 'X-tail' known from the state of the art was used (Tanaka et al., 1995, Biochem. Biophys. Res. Commun. 215, 744; und Rice, PCT/US 96/14033). The authentic non-translated region on the 5' end (=5' NTR) downstream of the T7 promoter was created by means of PCR, whereby an oligonucleotide, which corresponds to a shortened T7 promoter (TAA TAC GAC TCA CTA TAG) and the first 88 nucleotides of HCV, was used on one hand and one of the previously mentioned plasmids, which carries one of the 5' fragments of the genome, was used on the other hand. A complete HCV consensus genome was assembled from the subgenomic fragments with the smallest number of non-consensus replacements and inserted into a modified pBR322 vector. Deviations from the consensus sequence were

eliminated by means of site-directed mutagenesis. In order to produce run-off transcripts with an authentic 3' end, the 3' NTR of the isolates (with the end TGT) was modified to AGT (according to the sequence of the genotype 3 = clone 'WS' according to Kolykhalov et al., 1996, J. Virol. 70, 3363) and an additional nucleotide replacement was carried out at position 9562, to preserve the A:T pairing in the hairpin structure at the 3' end of the 3' NTR (Kolyhalov et al. ibid.). In order to eliminate an internal restriction site for the ScaI enzyme, a silent nucleotide replacement was further carried out. After joining the full-length genome with the appropriate 5' and 3' NTRs the complete HCV sequence was analyzed. No undesired nucleotide replacement was detected.

The HCV genome produced in this way should be hepatotropic according to the definition.

(B) Synthesis of selectable HCV subgenomic constructs

By means of the consensus genome described under (A), HCV subgenomic constructs were created, which include the antibiotic resistance gene neomycin phosphotransferase (NPT) and two sequences of internal ribosome entry sites (IRES). The biochemical procedures used for this are known and familiar to the expert (see: Sambrook, J., E.F. Fritsch, T. Maniatis, 1989, Molecularcloning: a laboratory manual, 2nd ed., Cold Spring Harbour Laboratory, Cold Spring Harbor, N.Y.; Ausubel et al. (eds.), 1994, Current Protocols in Molecular Biology, Vol. 1-3, John Wiley & Sons Inc., New York). The antibiotic resistance gene was inserted immediately after the 5' NTR, obtaining a bicistronic RNA as a result (see Fig. 1 A). However, the antibiotic resistance gene might as well be inserted at another site of the HCV subgenomic construct, for example within the nucleotide sequence encoding for the polyprotein, obtaining a monocistronic RNA as a result (see

Fig. 5 A), or in the 3' NTR (see Fig. 5 B). The IRES elements are one of the two HCV-IRES variants from nucleotides 1-377 or nucleotides 1-389 as well as the IRES of the encephalomyocarditis virus, which controls the translation of the HCV sequence downstream from the genes for NS2 or NS3 up to the authentic 3' end of the genome.

The two HCV-IRES variants mentioned were determined in the following way:

Based on deletion analyses of the 3' end of the HCV-IRES (Reynolds et al.1995, *EMBO J.* 14, 6010) various segments of the 5' NTR were fused with the NPT gene and analyzed with regard to the maximum number of colonies formed by means of co-transfection with a plasmid containing the T7 RNA polymerase gene. The best results were achieved with the HCV sequences from 1-377 to 1-389. Due to the fact that the AUG start codon of the HCV polyprotein is located at position 342 and is consequently included in the IRES sequence, 12 or 16 amino acids of the HCV core protein ("core protein") were fused with the neomycin phosphotransferase (see Fig. 1 A).

Accordingly, these modified HCV subgenomic constructs were given the designations I_{377}/NS2-3' (or I_{377}/NS3-3') and I_{389}/NS2-3' (or I_{389}/NS3-3'). They are illustrated in a diagram in Fig. 1A.

Different cell lines and primary cell cultures of human hepatocytes were transfected with in vitro transcripts of these modified parental HCV subgenomic constructs I_{377}/NS2-3' (or I_{377}/NS3-3') and I_{389}/NS2-3' (or I_{389}/NS3-3').

As a parallel negative control for all transfection experiments, an appropriately modified, but defective subgenome was constructed for every modified parental HCV subgenomic construct, which differs from the parental

construct due to the fact, that it has a deletion of 10 amino acids within the reading frame comprising the active site of the NS5B RNA polymerase (Behrens et al., 1996, *EMBO J.* 15, 12; and Lohmann et al., 1997, *J. Virol.* 71, 8416).

(C) Synthesis of selectable HCV genomic constructs

A NS2-3' subgenomic construct, linked at its 5' end to a fragment of the luciferase gene and the complete EMCV-IRES, was restricted with NcoI and SpeI and purified using preparative agarose gel electrophoresis. The vector obtained in this way was ligated with a NcoI/NotI-HCV fragment corresponding to the nucleotide positions 342 to 1968 of the HCV genome and a NotI/SpeI fragment corresponding to the nucleotide positions 1968-9605 in a 3-factor-ligation. The resulting construct, where the complete HCV open reading frame and the 3' NTR lie downstream from the luciferase gene fragment and the EMCV-IRES, was then restricted with PmeI and SpeI and ligated with the analogously restricted I_{389}/NS3-3'/wt subgenomic construct vector. This selectable HCV genomic construct is illustrated in Fig. 4.

(D) Production of in vitro transcripts corresponding to the HCV-RNA constructs

The previously described purified plasmid DNAs were linearized with ScaI and used for an in vitro transcription reaction after phenol/chloroform extraction and isopropanol precipitation using the following components: 80 mM HEPES, pH 7.5, 12,5 mM $MgCl_2$, 2 mM Spermidine, 40 mM Dithiothreitol, 2 mM of each NTP, 1 unit RNasin/µl, 50 µg/ml restricted DNA and approx. 2 units/µl T7 RNA polymerase. After 2 hrs. at 37 °C half of the amount of T7 polymerase was added and the reaction mixture was incubated for two further hours. In order to remove DNA the mixture was extracted with acid phenole (U. Kedzierski, J.C. Porte,

1991, Bio Techniques 10, 210), precipitated with isopropanol, the pellet was dissolved in water and incubated with DNase (2 units per µg DNA) for 60 min. at 37 °C. After subsequent extraction with acid phenole, acid phenole/chloroform and chloroform as well as isopropanol precipitation the dissolved RNA was quantified using optical density measurement and its integrity was checked using formaldehyde-agarose gel electrophoresis.

Example 2: Transfection experiments with the hepatoma cell line Huh 7

With all transfection experiments it was carefully ensured that any template DNA had been removed beforehand so as to avoid the possibility of this DNA integrating in transfected cells and conferring a neomycin resistance to them independent from HCV replication. The reaction mixture was therefore treated with 2 units of DNase per µg DNA for 60 min. at 37 °C after the in vitro transcription and extracted with acid phenole, acid phenole/chloroform and chloroform. Prior to being used for the transfection the precipitated RNA was analyzed using formaldehyde-agarose gel electrophoresis.

Three separate transfection experiments were carried out with the highly differentiated human hepatoma cell line Huh 7 (according to Nakabayashi et al. 1982, *Cancer Res.* **42**, 3858). Each time 15 µg RNA were introduced into 8 x 10^6 Huh 7 cells by electroporation and the cells were seeded in culture dishes with a diameter of 10 cm. 24 hours after seeding, neomycin (= G418) was added in a final concentration of 1 mg/ml. The culture medium was changed twice a week. After 3 - 5 weeks small colonies were visible, which were isolated and grown under the same culture conditions.

The cell clones obtained during the first experiment were isolated and subpassaged. Most of the clones died during this procedure, and the final yield was only 9 clones from cells, which had been transfected with the parental HCV subgenomic constructs and 1 clone (clone 8-1) from cells, which had been transfected with a defective HCV genomic construct, namely a defective NS2-3' HCV-RNA. Apart from an extended doubling time and the occasional occurrence of irregularly shaped cells, no consistent morphological differences were found between these 9 cell clones and the single cell clone (clone 8-1) or the parental Huh 7 cells.

The main criteria for functioning HCV genomic constructs are the formation of viral RNA with the correct size and the absence of (integrated) plasmid DNA, which could transfer or mediate on a G418 resistance.

To determine the HCV-RNA in the Huh 7 cells, the total RNA was isolated and analyzed by means of the common Northern Blot method using a plus-strand specific ribo probe (RNA probe). For this purpose the total RNA was isolated from the respective cell clones according to the method described by Chomczynski and Sacchi 1987, *Anal. Biochem.* 162, 156 and 10 µg RNA, which is equivalent to the total RNA content of 0,5 - 1 x 10^6 cells, are separated using denatured formaldehyde-agarose gel electrophoresis (lanes 3 to 12 of Fig. 1 B). At the same time 10^9 in vitro transcripts (ivtr.), which correspond to the I_{389}/NS2-3'/wt or the I_{389}/NS3-3'/wt replicon RNAs, are separated as well as size markers with authentic sequence (lane 1 or lane 2). The separated RNA was transferred onto nylon-based membranes and hybridized with a radioactively labeled plus-strand specific RNA probe, which was complementary to the complete NPT gene and the HCV-IRES of nucleotide 377 to nucleotide 1. The positions of the HCV specific RNAs (arrows) and the 28S rRNA are specified to the right of lane 12, the size (amount of nucleotides) of the RNA marker

is specified to left of lane 1. The RNA marker fragments contain HCV sequences and therefore hybridize with the ribo probe (= RNA probe). The results of this analysis are illustrated in Fig. 1 B.

With the exception of clone 8-1 transfected with the defective HCV genomic construct, all cell clones produced homogenous HCV-RNAs of correct lengths (approx. 8640 nucleotides in the case of the NS2-3' and approx. 7970 nucleotides in the case of NS3-3' replicon). This result is an indication of the fact, that the functional replicons or the functional HCV genomic constructs transfer the G418 resistance. In order to exclude, that the G418 resistance is caused by a plasmid DNA, which is integrated in the genome of the Huh 7 host cell and transcribed under control of a cellular promoter, the DNA of each clone was analyzed by means of a NPT gene specific PCR. Consequently the DNA was isolated from the selected Huh 7 cell clones by means of digestion with proteinase K (40µg/ml, 1h, 37 °C) in 10mMTris, pH7,5, 1mM EDTA, 0,5 % SDS and subsequent extraction with phenol, phenol/chloroform and isopropanol precipitation. The DNA precipitate was dissolved in 10 mM Tris (pH 7,5) and 1 mM EDTA and incubated with Rnase A for 1 hour. Following a phenol/chloroform extraction and ethanol precipitation, 1 µg DNA, equivalent to 4 - 8 x 10^4 cells, was analyzed by means of PCR using NPT gene specific primers (5'-TCAAGACCGACCTG TCCGGTGCCC-3' and 5'-CTTGAGCCTGGCGAACAGTTCGGC-3'), and a DNA fragment consisting of 379 nucleotides was generated. The specificity of the PCR product was established by means of the Southern Blot method, in which a DNA fragment labeled by digoxigenin was used, which corresponds to the NPT gene. As positive controls (for the detection of possibly contaminating nucleic acids) the PCR method was carried out with 10^7 plasmid molecules or 1 µg DNA from a BHK cell line, which was stably transfected with a neomycin resistance gene, and

as negative control, the PCR was carried out with the same reagents but without added DNA.

The results of this analysis are illustrated in Fig. 1 C. The lanes 1 and 2 represent the positive controls, lane 13 represents the negative control. The numbers to the left of lane 1 indicate the sizes of the nucleotide marker molecules.

A NPT-DNA could not be detected in any cell clone, apart from clone 7-3 (Fig.1C, lane 3), which was obtained from cells after transfection with a NS2-3' replicon/NS2-3'HCV genomic construct, and clone 8-1 (Fig.1C, lane 12), which was obtained from cells after transfection with a defective HCV genomic construct. This result was another indication of the fact, that the G418 resistance of most clones was passed on by the replicating HCV-RNA. But even regardless of these results, it is unlikely, that integrated plasmid DNA produces HCV-RNAs of correct sizes, because the plasmids used for the in vitro transcription contain neither an eukaryotic promoter nor a polyadenylation signal. In the case of clone 7-3 the resistance is therefore very probably passed on by the HCV-RNA construct or the replicating HCV-RNA as well as by an integrated NPT DNA sequence, whereas the resistance of the cells of clone 8-1 is only caused by the integrated plasmid DNA.

Clone 9-13 (Fig. 1 B, lane 11) was subject to further tests, to confirm that the G418 resistance was passed on by a self-replicating HCV-RNA. Clone 8-1, which carries the integrated copies of the NPT gene, was used throughout as negative control. A PCR was carried out, which allows the detection of < 1000 NPT gene copies in ~ 40.000 cells, with the aim to rigorously exclude the presence of NPT-DNA in clone 9-13. The result of this PCR is illustrated in Fig. 2A. The PCR proceeded in detail as follows:

During the test, 10^6 - 10^2 plasmid molecules (I_{377}/NS3-3'/wt) were used either directly (lanes 7 - 11) or after adding each 1 µg 9-13 DNA (lanes 2 - 6). The specificity of the amplified DNA fragment was determined by Southern Blot using a NPT specific probe. A PCR without DNA probe was carried out as negative control (lane 12).

Even with this sensitive method, no plasmid DNA could be detected in one µg DNA of the cell clone 9-13 (lane 1). To estimate the amount of HCV plus- and minus-strand RNAs in these cells, a dilution series of total RNA was analyzed with the Northern Blot method using a plus- or minus-strand specific radioactively labeled ribo probe (= RNA probe). For this purpose, 8, 4 or 2 µg of total RNA, which have been isolated from the cell clones 9-13 and 8-1, were analyzed in parallel to known amounts of in vitro transcripts with plus- or minus-strand polarity (control RNAs) in the Northern Blot method and were then subjected to a hybridization. The hybridization was carried out with a plus-strand specific ribo probe, which covered the complete NPT gene and the HCV-IRES ('plus-strand', top panel), or with a minus-strand specific RNA probe, which was complementary to the NS3 sequence ('minus-strand', bottom panel). The arrows mark the positions of replicon RNA. The results of this analysis are illustrated in Fig. 2 B.

Approx. 10^8 copies/µg total RNA were detected in the case of the plus-strand, which is equivalent to 1000 - 5000 HCV-RNA molecules per cell, whereas the amount of minus-strand RNA was 5- to 10-times less. This result corresponds to the assumption, that the minus-strand RNA is the replicative intermediate form or intermediate copy, which is used as a template for the synthesis of the plus-strand molecules.

Due to the fact that the reaction is mainly catalyzed by the viral RNA-dependent RNA polymerase, the synthesis of

the HCV-RNAs should be resistant to dactinomycin, an antibiotic, which selectively inhibits the RNA synthesis of DNA templates, but not the RNA synthesis from RNA templates. To confirm this assumption, cells were incubated with [^{3}H] uridine in the presence of dactinomycin, the radioactively labeled RNAs were extracted, separated by means of denaturing agarose gel electrophoresis and analyzed with the aid of a common Bio-Imager using a [^{3}H]-sensitive screen. For this purpose approx. 5 x 10^5 cells of the clones 9-13 and 8-1 were incubated at a time with 100 µ Ci [^{3}H] uridine for 16 hrs. in the absence (-) or presence (+) of 4 µg/ml of dactinomycin (Dact). Following this labeling reaction the total RNA was prepared and analyzed by means of formaldehyde-agarose gel electrophoresis. Only 1/10 of the total RNA is illustrated in the first two lanes. The radioactively labeled RNA was visualized using a BAS-2500 Bio-Imager (Fuji).

The results of this analysis are illustrated in Fig. 2 C. In accordance with the inhibitor profile of the NS5B polymerase (Behrens et al., 1996, *EMBOJ.* 15, 12 and Lohmann et al., 1997, *J. Virol.* 71, 8416), dactinomycin had no influence on the replication of the HCV RNA, whereas the synthesis of cellular RNA was inhibited. A RT-PCR was carried out for recloning the replicating sequences, to confirm the identity of the viral RNA. The sequence analysis of the recloned RNA showed that the RNA in clone 9-13 is HCV specific and corresponds to the transfected transcript of the HCV construct I_{377}/NS3-3'/wt.

For the analysis of the viral proteins, at first the respective cells were metabolically radioactively labeled with [^{35}S] methionine/cysteine, then lysed and afterwards the HCV specific proteins were isolated from the cell lysates by means of immunoprecipitation. The results of these analyses are illustrated in Fig. 3 A. The detailed procedure was as follows: Cells of the cell clones 9-13

(wt) and 8-1 (Δ) were metabolically radioactively labeled by treating them with a protein labeling mixture familiar to the expert and available on the market (for example. NEN Life Science). The HCV specific proteins were separated from the cell lysate by immunoprecipitation (IP) under non-denaturing conditions (for example according to Bartenschlager et al., 1995, *J. Virol.* 69, 7519) using three different antisera (3/4, 5A, 5B, according to the labeling on the top end of the lanes 1 to 12). The immune complexes were analyzed by means of tricine SDS-PAGE and made visible by means of autoradiography. To obtain authentic size markers, the homologous replicon construct I_{377}/NS3-3'/wt was subject to a transient expression by the vaccinia virus-T7 hybrid system in the Huh 7 cells. The resulting products were used as size markers (lanes 7 - 9) parallel to the cells of the clones 9-13 and 8-1. Identified HCV proteins are labeled on the left edge of lane 1, the molecular weights (in Kilodalton) are specified on the right edge of lane 9. It should be noted that the NS3/4 specific antiserum ('3/4') used preferably reacts with NS4A and NS4B causing an underrepresentation of NS3.

All viral antigens could unambiguously be detected, and their apparent molecular weights did not show any difference to those being detected after transient expression of the same bicistronic HCV-RNA construct in the original Huh 7 cells. An immunofluorescence detection reaction was carried out using NS3 and NS5A specific antisera, to determine the subcellular distribution of the viral antigens (for example according to Bartenschlager et al., 1995, *J. Virol.* 69, 7519). For this purpose cells of the clones 9-13 (wt) and 8-1 (Δ) were fixed with methanol/acetone 24 hrs. after incubating on coverslips and incubated with polyclonal NS3 or NS5A specific antisera. The bound antibodies were made visible with a commercially available FITC conjugated anti-rabbit antiserum. The cells

were counterstained with the 'Evans Blue' stain to suppress unspecific fluorescence signals.

The results of this detection test are illustrated in Fig. 3 B. A strong fluorescence in the cytoplasm could be detected with both antisera. The NS5A specific antiserum also caused a slight nuclear fluorescence, which indicates that at least small amounts of this antigen also reach the nucleus. But the generally dominating presence of the viral antigens in the cytoplasm are a strong indication that HCV-RNA replication occurs in the cytoplasm, as is the case with most RNA viruses.

These results prove clearly that the establishment of a cell culture system for the HCV could be accomplished with the test arrangement described, the efficiency of which surpasses everything known up until now by far and for the first time allows the detection of viral nucleic acids and proteins with conventional and approved biochemical methods. This efficiency actually allows detailed examination of HCV pathogenesis, genetic analyses of different HCV functions and a precise study of the virus/host cell interaction, through which new starting points for the development of a antiviral therapy can be defined.

Example 3: Transfection of Huh 7 cells with HCV total constructs

Huh7 cells are transfected and selected as described in example 2, whereby in this case selectable constructs are used, which contain the complete virus genome. Corresponding to Example 2, the resulting cell clones are tested for the absence of HCV-DNA by means of PCR and the productive replication of HCV-RNA is then established by means of Northern Blot, [^{3}H]uridine labeling in the

presence of dactinomycin, detection of the viral proteins or antigens preferably with the aid of the Western Blot, the immunoprecipitation or immunofluorescence. In contrast to the arrangements described in Example 2, the construct described here makes it possible to obtain more complete and very likely infectious viruses, which has not been the case in the subgenomic constructs described in Example 2. These viruses existing in the cell and the cell culture supernatant are concentrated for example by means of ultracentrifugation, immunoprecipitation or precipitation with polyethyleneglycol, and all exogenous nucleic acids, which means those that are not incorporated into the virus particle, are digested by incubation with nucleases (RNase, DNase, micrococal nuclease). In this way all contaminating nucleic acids, which are not included in the protecting virus particle, can be removed. After inactivation of the nucleases, the protected viral RNA is isolated for example by means of incubation with proteinase K in a buffer containing SDS, by extracting with phenol and phenol/chloroform and detected by means of Northern Blot or RT-PCR using HCV specific primers. Also in this test arrangement, the combination of the HCV consensus genome described with a selection marker was crucial for the efficient production of viral RNA, viral protein and therefore HCV particles.

Example 4: Production and application of a HCV-RNA construct, whereby the resistance gene is linked to the HCV subgenomic sequence via a ribozyme or a recognition site for a ribozyme.

A HCV-RNA construct is produced according to Example 1 or Example 3, where an antibiotic resistance gene is linked to the HCV-RNA sequence through a ribozyme or a recognition sequence for a ribozyme. These constructs are illustrated in a diagram in Fig. 7. Huh 7 cells are transfected with

this HCV-RNA construct as described in Example 2. A selection with the appropriate antibiotic follows the transfection into the cells. The inserted ribozyme is activated in the cell clones obtained in the procedure or, in the case of a construct, which carries a recognition sequence for a ribozyme, the ribozyme is transfected into the cell (for example by means of transfection of a ribozyme construct or infection with a viral expression vector, into which the respective ribozyme has been inserted). In both cases the resistance gene is separated from the HCV-RNA sequence by the ribozyme-dependent cleavage. The result in the case of the HCV genome is an authentic HCV genome without a resistance gene, which can form authentic infectious virus particles. A HCV replicon without resistance gene is created in the case of the HCV subgenomic constructs.

Example 5: Co-transfection of a HCV-RNA construct with a separate luciferase transfection construct

A HCV-RNA construct is produced according to Example 1 (A) or Example 3 or Example 4. At the same time a transfection construct is produced, which comprises the luciferase gene, whereby this luciferase gene is linked to a first nucleotide sequence, which encodes a HCV protease (for example NS3 protease) cleavage site, to a second nucleotide sequence, which encodes for another protein or a part of another protein. HCV-RNA construct and transfection construct are transfected into any host cells, preferably hepatoma cells, most preferably Huh 7 cells. This can be realised as described in Example 2. The product of the modified luciferase gene is a luciferase fusion protein, where the luciferase is inactivated due to the fusion with the foreign part. The fusion protein, which contains a recognition sequence for a HCV protease, is cleaved in transfected cells with high HCV replication, and

consequently the active form of the luciferase, which can be identified through luminometric measurement, is released. If the replication of the HCV-RNA construct is inhibited the fusion protein will not be cleaved and no active luciferase will be released. The quantitative determination of the luciferase is therefore a measure for the replication of the HCV construct. Instead of the luciferase gene, another reporter gene can just as easily be used, which is modified in the same way, so that its expression depends on the viral replication, although this reporter gene is not part of the HCV construct. A cellular protein, which is deactivated or activated by the HCV proteins or nucleic acid, can also be used as a so called surrogate marker. The expression or activity of this surrogate marker is in this case a measure for the replication of the viral DNA.

Example 6: Production of HCV subgenomic constructs with integrated foreign genes to be used as liver cell specific vector in gene therapy

These recombinant and selectable HCV subgenomic constructs are transfected in transcomplementing helper cell lines, which means in cell lines, which express the missing functions (for example the structural proteins) in an inducible or constitutive way. Cell clones containing a functional HCV subgenomic construct can be established through appropriate selection. The viral structural proteins expressed from the host cell allow the formation of virus particles, into which the RNA of the HCV subgenomic constructs will be transfected. The results are therefore virus-like particles, which contain a HCV subgenomic construct according to the invention including the inserted foreign gene and which can transmit this to other cells by means of infection. An example for this construct is illustrated in Fig. 8.

It is also possible to use the HCV subgenomic construct with integrated foreign gene directly as an expression vector. This involves the same method as mentioned previously except that cell lines, which do not express transcomplementing factors, are transfected. In this case the HCV construct is only used as an expression vector.

Example 7: Production of cell culture adapted HCV-RNA constructs

(A) Method of isolation

The following method was used to determine adaptive mutations and to produce cell culture adapted HCV-RNA constructs: cells were transfected with a HCV-RNA construct as described in Examples 1 and 2 and G418-resistant cell clone produced. For the determination of ability to replicate (understood in this context to be the number of G418 resistant cell clones obtained per microgram of transfected HCV-RNA or HCV-RNA construct), the total RNA from one of the cell clones, [the 9-13 clone (Fig. 1 B, lane 11)], was isolated and the quantity of HCV-RNA contained within it was determined by Northern Blot as described in Fig. 2 B. Ten micrograms of the total RNA, containing approx. 10^9 molecules of HCV-RNA, was then transfected into naive Huh-7 cells using electroporation (Fig. 9). In parallel, 10^9 in vitro transcripts of the analoguous *neo*-HCV-RNA, which had been adjusted with isolated total RNA from naive Huh-7 cells to a total RNA quantity of 10 µg, were transfected in naive Huh-7 cells. After selection with G418, the number of cell colonies, expressed as 'colony forming units (cfu) per microgram RNA', was determined in both these cultures. At a concentration of 500 µg/ml G418 in the selection medium, the number of colonies obtained with the HCV-RNA contained

in *isolated* total RNA from clone 9-13, was approx. 100,000 cfu per microgram HCV-RNA. In contrast, only 30 - 50 colonies were obtained with the same quantity of in vitro transcribed HCV-RNA. This result confirms that the specific infectivity of the HCV-RNA isolated from the cell clones is approx. 1,000 - 10,000 times higher than the infectivity of the analoguous in vitro transcripts. The experimental approach is shown in Fig. 9.

With the aid of 'long-distance RT-PCR', the HCV-RNA was amplified from the total RNA of the 9-13 cells, the PCR amplificate was cloned and numerous clones were sequenced. A comparison of the sequences of these recloned RNAs with the sequence of the RNA originally transfected into the naive Huh-7 cells, showed that the recloned RNAs possessed numerous amino acid exchanges distributed over the whole HCV sequence (Fig. 10). *Sfi*I fragments of these recloned mutants were used to replace the analoguous *Sfi*I fragment of the original replicon construct, and RNAs of the respective mutants were transfected in naive Huh-7 cells. After selection with G418 the number of colonies created was determined for each HCV-RNA mutant. While only 30 - 50 colonies per microgram RNA were obtained with the parental RNA the number of colonies was noticeably higher for two of the recloned variants (Fig. 10). In the case of the HCV-RNA constructs 9-13I and 9-13C the specific infectivity was increased to 100 - 1,000 cfu per microgram RNA and for 9-13F replicon it was 1,000 - 10,000 cfu per microgram RNA. These results show that the amino acid exchanges in the analysed NS3-5B regions of the mutants 9-13I, 9-13C and particularly of 9-13F, led to a considerable increase in ability to replicate. In contrast all the other HCV-RNA constructs (9-13 A, B, G, H und K) were no longer able to replicate, they thus contained lethal mutations.

In order to answer the question which of the amino acid exchanges in the 9-13F construct led to an increase in

replication, the exchanges were introduced separately or in combination into the parental HCV-RNA construct, and the corresponding RNAs transfected in naive Huh-7 cells. The result of the transfection with these RNAs is summarised in Table 1. From this it is evident that in the present example the high ability to replicate is determined by several mutations. The amino acid exchanges in the HCV-RNA regions NS5A and NS4B make the greatest contribution. The single exchanges in the NS3-Region also make a contribution and perhaps they are synergistic.

These results confirm that it was through the G418 selection of the cells transfected with the *neo*-HCV-RNA construct that there was enrichment of those HCV-RNAs having noticeably higher ability to replicate. HCV-RNA constructs with greatly differing replication efficiencies can be selected using the experimental approach described here. The higher the concentration of the antibiotic in the selection medium, in/on which the HCV-RNA construct containing cells are cultivated for selection, the higher must be the extent of adaptive mutations and hence replication efficiency of the relevant HCV-RNA constructs, to allow the cells to grow under these conditions. If the selections are carried out using lower antibiotic concentrations, cells can survive and multiply, but the HCV-RNA construct shows a comparatively lower replication efficiency and fewer adaptive mutations.

As has been shown, the 9-13F HCV-RNA construct described so far, which contains several adaptive mutations, had a higher replication efficiency than the parental HCV-RNA. In order to obtain HCV-RNAs with even higher replication in cell culture, the HCV-RNA contained in the total RNA of a selected cell clone was passaged several times in naive Huh-7 cells. The selected 5-15 cell clone, was obtained by transfection with the HCV-RNA construct I_{389}/NS3-3' (Fig. 1). It largely corresponds to the cell clone 9-13, produced

by transfection with a HCV-RNA construct, having a HCV-IRES shorter by 22 nucleotides (I_{377}/NS3-3'; Fig. 1). Ten micrograms of total RNA, isolated from cell clone 5-15, were transfected into naive Huh-7 cells using electroporation and the cells subjected to a selection with 1 mg/ml G418. The total RNA from one of the cell clones thus produced was again isolated, transfected into naive Huh-7 cells and selected in the same way. This process was repeated a total of four times. After the fourth passage the total RNA was isolated from a cell clone and the *neo*-HCV-RNA amplified with the aid of the 'long-distance RT-PCR'. The amplified DNA fragment was digested with the restriction enzyme *Sfi*I and inserted into the *Sfi*I-restricted parental construct I_{389}/NS3-3'. Over 100 DNA clones were obtained altogether and then analysed by means of restriction digestion. In vitro transcribed RNA of about 80 of these clones was each transfected into naive Huh-7 cells and subjected to a selection with 500mg/ml G418. Of the 80 neo-HCV-RNA variants examined, the great majority proved to be replication defective. However, the specific infectivity, expressed as 'colony forming units' per microgram RNA, was noticeably increased in the case of two mutants, 5.1 and 19 (Table 2). Through several passages of the RNA in cell culture it is clear that HCV-RNAs are produced whose replication efficiency due to mutations („adaptive mutations") is several orders of magnitude higher than the original RNA cloned from patients.

(B) Modified method

Adaptive mutations produced and identified according to (A) can be transferred into a HCV-RNA construct with low ability to replicate. This leads to a huge increase in the replication of this construct. The increase is so great it can be demonstrated that HCV-RNAs transfected into cell culture can replicate even in the absence of selection pressure Fig. 12 shows a comparison of the replication

efficiency of HCV-RNAs, which corresponded either to the starting sequence or to the adaptive sequences 9-13F or 5.1. For the purposes of simple measurement, the *neo*-gene was removed and replaced by the gene for luciferase. The negative control used was again a HCV-RNA construct that was replication defective due to a deactivating mutation in the NS5B RNA polymerase. Already 24 hours after transfection a noticeable difference is evident in luciferase activity between the defective RNA and the 9-13F or 5.1 constructs, while hardly any difference could be seen between the defective RNA (318 DN) and the parental RNA construct (wt) that possessed no adaptive mutations. During the whole period of observation, the highest luciferase activity, and thus highest replication, was obtained with the 5.1-RNA. These results not only confirm the high replication efficiency of this RNA, but also show that it is possible to create a cell culture system with adapted HCV-RNA constructs for which the presence of a selectable gene is no longer necessary. A summary of the nucleotide and amino acid differences between the starting construct and the mutants 9-13F, 5.1 and 19 is presented in Table 3.

Example 8: Production of cell culture adapted HCV-RNA full-length genome

In the examples 1 to 7 a subgenomic HCV-RNA was used which lacked the whole structural protein region from core up to p7 or even NS2. It will be shown in this example that it is possible to make a HCV full-length genome replicate in cell culture with the aid of an adapted NS3-5B sequence. For this purpose the *SfiI* fragment of the highly adapted HCV-RNA 5.1 produced according to Example 7 is first transferred into a selectable HCV full-length genome (Fig. 12). This HCV genome was transfected into naive Huh-7 cells and subjected to selection with various G418

concentrations. Depending on the strength of selection (the G418 concentration), a varying large number of cell clones was obtained (Fig. 12 B). By contrast no colonies were obtained with the parental HCV full-length genome containing no adaptive mutations, as was the case for the negative control, which was replication defective due to a deactivating mutation in the NS5B RNA polymerase. To confirm that the thus resulting cell clones really contained an autonomously replicating HCV full-length construct, total RNA from several cell clones was isolated and analysed by means of the Northern Blot method. The full-length HCV-RNA was clearly detectable in all cell clones (Fig. 12). It is thus clearly confirmed, that with the aid of cell culture adapted HCV sequences it is possible to produce a HCV full-length genome, which replicates highly efficiently and autonomously in a cell line, i.e. adapted HCV full-length genomes can also be produced with the system of the invention. Furthermore, as this clone possesses the complete HCV sequence, i.e. it also possesses the structural proteins necessary for virus particle formation, it is possible to produce large quantities of infectious virus particles in cell cultures with this system. As a confirmation of these viruses, cell-free cell supernatants carrying a replicating HCV full-length genome, are added to naive Huh-7 cells and the thus infected cells subjected to selection with G418. Each cell clone growing under these conditions originates from an infected cell. The viruses in the cell culture supernatant of cells possessing a replicating HCV full-length genome can be enriched and purified using various known state of the art methods such as ultracentrifugation or microdialysis. They can then be used for the infection of naive cells. Using this method it is clearly demonstrated that cell culture adapted full-length genomes can be produced with the HCV cell culture system of the invention. These genomes replicate with high efficiency in cells and produce infectious viruses. The latter can be detected by

infection of an experimental animal, preferably a chimpanzee.

Example 9: Production of HCV full-length constructs and HCV subgenomic constructs with reporter genes

A HCV-RNA construct is produced in which a reporter gene is inserted in place of the antibiotic resistance gene (Fig. 13). Replication can thereby be determined through the quantity or activity of the reporter gene or reporter gene product. The reporter gene is preferably a gene from the group of the luciferase genes, the CAT gene (chloramphenicol acetyl transferase gene), the lacZ gene (beta galactosidase gene), the GFP gene (green fluorescence protein gene), the GUS gene (glucuronidase gene) or the SEAP gene (secreted alkaline phosphatase gene). This reporter gene and its products, namely the relevant reporter proteins, can be detected for example using fluorescence, chemiluminescence, colorimetrically or by means of immunological methods (for example, enzyme-linked immunosorbent assay, ELISA).

The reporter gene can be expressed either from a separate IRES or in the form of a fusion protein, which is active either as such or fused with a HCV protein via a poteolytically cleavable amino acid sequence in such a way that the reporter is separated from the HCV protein by cleavage of a cellular or viral (HCV) protease.

Example 10: Production of HCV full-length constructs with integrated foreign genes used as liver cell specific vectors for gene therapy or as expression vectors.

The construct (Fig. 14) is transfected in cells and leads to the formation of HCV virus particles that can be used

for the infection of further cells. Since the virus particles have encapsidated RNA with a foreign gene, it can be used in the infected cells for the production of the protein coded by this foreign gene. Cells transfected with the construct also express the foreign gene.

Example 11: Production of monocistronic HCV-RNA constructs in which the resistance gene product is expressed as a fusion protein with the HCV portion.

It is an advantage for some tests if the HCV-RNA construct does not possess a heterologous IRES element. Tests of this type are, for example, the determination of interferon resistance. If a cell possessing a HCV-RNA construct is incubated with interferon alpha or beta, a reduction in replication of the HCV-RNA results. In order to explain the mechanism of this effect it is necessary for the HCV-RNA construct not to possess any heterologous IRES, as otherwise it is not possible to determine whether the interferon mediated inhibition is via inhibition of the HCV replication or inhibition of the heterologous IRES. For this reason constructs are produced for which the resistance gene is fused with a HCV protein (Fig. 15). Either the fusion protein is active as such or the resistance gene product is linked to a HCV protein via a proteolytically cleavable amino acid sequence in such a way that it is separated from the HCV protein by a cellular or viral (HCV) protease.

Table 1: Specific infectivities (cfu/µg RNA) of HCV RNA constructs with adaptive mutations found with the 9-13F mutant introduced into the parental construct I_{389}/NS3-3'/wt

amino acid exchange[1]	HCV protein	cfu/µg RNA[2]
none		30 - 60
1283 arg -> gly	NS3	200 - 250
1383 glu -> ala	NS3	30 - 60
1577 lys -> arg	NS3	30 - 60
1609 lys -> glu	NS3	160 - 300
(1283 arg -> gly + 1383 glu -> ala + 1577 lys -> arg + 1609 lys -> glu)	NS3	360 - 420
1936 pro -> ser	NS4B	1000-5000
2163 glu -> gly	NS5A	1000-5000
2330 lys -> glu	NS5A	30 - 60
2442 ile -> val	NS5A	30 - 60
all together		5000

[1] amino acid change in the polyprotein of the HCV isolate con 1 (EMBL-gene bank No. AJ238799); amino acids are given in single letter code.

[2] Colony forming units (number of cell clones) obtained with a selection of 500 µg/ml G418.

Table 2: Specific infectivities (cfu/µg RNA) of the parental HCV RNA construct I_{389}/NS3-3'/wt and the variants 9-13I, 9-13F, 5.1 and 19.

Transfected RNA variant	cfu/µg RNA[1]
wild type	30 - 50
9-13 I	100 - 1.000
9-13 F	1.000 - 10.000
5.1	50.000 - 100.000
19	50.000 - 100.000

[1] Colony forming units (number of cell clones) obtained with a selection of 500µg/ml G418.

Table 3: Nucleotide and amino acid sequence differences between the parental HCV RNA construct I_{389}/NS3-3'/wt and the mutants 9-13I, 9-13F, 5.1 und 19

Clone	nt-position	nt-exchange	aa-exchange
9-13 I	3685	C > T	P > L
	4933	C > T	T > M
	5249	T > C	-
	8486	C > T	-
	8821	G > A	W > stop
	8991	C > G	R > G
	9203	A > G	-
	9313	T > C	F > S
	9346	T > C	V > A
9-13 F	3866	C > T	-
	4188	A > G	R > G
	4489	A > C	E > A
	4562	G > A	-
	4983	T > C	-
	5071	A > G	K > R
	5166	A > G	K > E
	6147	C > T	P > S
	6829	A > G	E > G
	7329	A > G	K > E
	7664	A > G	I > V
	8486	C > T	-
	8991	C > G	R > G
NK5.1	4180	C > T	T > I
	4679	C > T	-
	4682	T > C	-
	5610	C > A	L > I
	6437	A > G	-
	6666	A > G	N > D

	6842	C > T	-
	6926	C > T	-
	6930	T > C	S > P
	7320	C > T	P > S
	7389	A > G	K > E
NK19	3946	A > G	E > G
	4078	C > G	A > G
	4180	C > T	T > I
	4682	T > C	-
	5610	C > A	L > I
	5958	A > T	M > L
	6170	T > A	-
	6596	G > A	-
	6598	C > G	A > G
	6833	C > T	-
	6842	C > T	-
	6930	T > C	S > P
	7141	A > G	E > G
	7320	C > T	P > S
	7389	A > G	K > E
	7735	G > A	S > N

Given are the differences between the nucleotide and amino acid sequences of the parental HCV RNA sequence con 1 (EMBL-gene bank No. AJ238799) and those of the cell culture adapted HCV RNAs. Numbers refer to the nucleotide and amino acid positions of the con 1 isolate. nt, nucleotide; aa, amino acid.

SEQUENCE LISTING

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Vainberg, Irina

Fr m: Fehlner, Paul
Sent: Tuesday, September 30, 2003 8:06 AM
Tq: 'Michael Sasaki, Esq. (msasaki@anadyspharma.com)'
Cc: Vainberg, Irina
Subject: Hepatitis C Virus Constructs ... (PCT/US01/46350; our file 00342/200h395-WO0)

Michael:

In the absence of instructions to the contrary, as requested in our email of September 26, we have instructed the Authorized Office to go ahead with issuing an International Preliminary Examination Report. We will forward the report to you in due course.

Best regards, Paul

Paul F. Fehlner, Ph.D.
Darby & Darby P.C.
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New York, NY 10022

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